

PATENT COOPERATION TREATY

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

From the INTERNATIONAL BUREAU

To:

Commissioner
US Department of Commerce
United States Patent and Trademark
Office, PCT
2011 South Clark Place Room
CP2/5C24
Arlington, VA 22202
ETATS-UNIS D'AMERIQUE
in its capacity as elected Office

Date of mailing (day/month/year) 08 May 2001 (08.05.01)	
International application No. PCT/US00/18325	Applicant's or agent's file reference 55859-015
International filing date (day/month/year) 03 July 2000 (03.07.00)	Priority date (day/month/year) 01 July 1999 (01.07.99)
Applicant ROKITA, Steven, E. et al	

1. The designated Office is hereby notified of its election made:

☒ in the demand filed with the International Preliminary Examining Authority on:
31 January 2001 (31.01.01)

☐ in a notice effecting later election filed with the International Bureau on:

2. The election ☒ was
☐ was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

BEST AVAILABLE COPY

PCT

**NOTICE INFORMING THE APPLICANT OF THE
COMMUNICATION OF THE INTERNATIONAL
APPLICATION TO THE DESIGNATED OFFICES**

(PCT Rule 47.1(c), first sentence)

From the INTERNATIONAL BUREAU

To:

KIM, Joseph, H.
McDermott, Will & Emery
600 13th Street, NW
Washington, DC 20005-3096
ETATS-UNIS D'AMERIQUE

JAN 23 2001

McDermott, Will & Emery

Date of mailing (day/month/year) 11 January 2001 (11.01.01)		IMPORTANT NOTICE	
Applicant's or agent's file reference 55859-015			
International application No. PCT/US00/18325	International filing date (day/month/year) 03 July 2000 (03.07.00)	Priority date (day/month/year) 01 July 1999 (01.07.99)	
Applicant UNIVERSITY OF MARYLAND, COLLEGE PARK et al			

1. Notice is hereby given that the International Bureau has communicated, as provided in Article 20, the international application to the following designated Offices on the date indicated above as the date of mailing of this Notice:
AU,KP,KR,US

In accordance with Rule 47.1(c), third sentence, those Offices will accept the present Notice as conclusive evidence that the communication of the international application has duly taken place on the date of mailing indicated above and no copy of the international application is required to be furnished by the applicant to the designated Office(s).

2. The following designated Offices have waived the requirement for such a communication at this time:

**AE,AL,AM,AP,AT,AZ,BA,BB,BG,BR,BY,CA,CH,CN,CR,CU,CZ,DE,DK,DM,EA,EE,EP,ES,FI,GB,GD,
GE,GH,GM,HR,HU,ID,IL,IN,IS,JP,KE,KG,KZ,LC,LK,LR,LS,LT,LU,LV,MA,MD,MG,MK,MN,MW,MX,
NO,NZ,OA,PL,PT,RO,RU,SD,SE,SG,SI,SK,SL,TJ,TM,TR,TT,TZ,UA,UG,UZ,VN,YU,ZA,ZW**

The communication will be made to those Offices only upon their request. Furthermore, those Offices do not require the applicant to furnish a copy of the international application (Rule 49.1(a-bis)).

3. Enclosed with this Notice is a copy of the international application as published by the International Bureau on
11 January 2001 (11.01.01) under No. WO 01/02370

REMINDER REGARDING CHAPTER II (Article 31(2)(a) and Rule 54.2)

If the applicant wishes to postpone entry into the national phase until 30 months (or later in some Offices) from the priority date, a demand for international preliminary examination must be filed with the competent International Preliminary Examining Authority before the expiration of 19 months from the priority date.

It is the applicant's sole responsibility to monitor the 19-month time limit.

Note that only an applicant who is a national or resident of a PCT Contracting State which is bound by Chapter II has the right to file a demand for international preliminary examination.

REMINDER REGARDING ENTRY INTO THE NATIONAL PHASE (Article 22 or 39(1))

If the applicant wishes to proceed with the international application in the national phase, he must, within 20 months or 30 months, or later in some Offices, perform the acts referred to therein before each designated or elected Office.

For further important information on the time limits and acts to be performed for entering the national phase, see the Annex to Form PCT/IB/301 (Notification of Receipt of Record Copy) and Volume II of the PCT Applicant's Guide.

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland Facsimile No. (41-22) 740.14.35	Authorized officer J. Zahra Telephone No. (41-22) 338.83.38
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PATENT COOPERATION TREATY

PCT

INFORMATION CONCERNING ELECTED
OFFICES NOTIFIED OF THEIR ELECTION

(PCT Rule 61.3)

From the INTERNATIONAL BUREAU

To:

RECEIVED

MAY 21 2001

KIM, Joseph, H.
 McDermott, Will & Emery
 600 13th Street, NW
 Washington, DC 20005-3096
 ETATS-UNIS D'AMERIQUE

Date of mailing (day/month/year) 08 May 2001 (08.05.01)		
Applicant's or agent's file reference 55859-015		IMPORTANT INFORMATION
International application No. PCT/US00/18325	International filing date (day/month/year) 03 July 2000 (03.07.00)	
Priority date (day/month/year) 01 July 1999 (01.07.99)		
Applicant UNIVERSITY OF MARYLAND, COLLEGE PARK et al		

1. The applicant is hereby informed that the International Bureau has, according to Article 31(7), notified each of the following Offices of its election:

AP : GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW

EP : AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE

National : AU, BG, CA, CN, CZ, DE, IL, JP, KP, KR, MN, NO, NZ, PL, RO, RU, SE, SK, US

2. The following Offices have waived the requirement for the notification of their election; the notification will be sent to them by the International Bureau only upon their request:

EA : AM, AZ, BY, KG, KZ, MD, RU, TJ, TM

OA : BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG

National : AE, AL, AM, AT, AZ, BA, BB, BR, BY, CH, CR, CU, DK, DM, EE, ES, FI, GB, GD, GE, GH,
 GM, HR, HU, ID, IN, IS, KE, KG, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MW, MX, PT, SD,
 SG, SI, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW

3. The applicant is reminded that he must enter the "national phase" before the expiration of 30 months from the priority date before each of the Offices listed above. This must be done by paying the national fee(s) and furnishing, if prescribed, a translation of the international application (Article 39(1)(a)), as well as, where applicable, by furnishing a translation of any annexes of the international preliminary examination report (Article 36(3)(b) and Rule 74.1).

Some offices have fixed time limits expiring later than the above-mentioned time limit. For detailed information about the applicable time limits and the acts to be performed upon entry into the national phase before a particular Office, see Volume II of the PCT Applicant's Guide.

The entry into the European regional phase is postponed until 31 months from the priority date for all States designated for the purposes of obtaining a European patent.

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland Facsimile No. (41-22) 740.14.35	Authorized officer: F. Baechler Telephone No. (41-22) 338.83.38
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PATENT COOPERATION TREATY

RECEIVED

PCT

FEB 23 2001

NOTIFICATION CONCERNING
THE FILING OF AMENDMENTS OF THE CLAIMS
(PCT Administrative Instructions, Section 417)

From the INTERNATIONAL BUREAU

To:

KIM, Joseph, H.
McDermott, Will & Emery
600 13th Street, NW
Washington, DC 20005-3096
ETATS-UNIS D'AMERIQUE

Date of mailing (day/month/year) 13 February 2001 (13.02.01)	IMPORTANT NOTIFICATION
Applicant's or agent's file reference 55859-015	
International application No. PCT/US00/18325	International filing date (day/month/year) 03 July 2000 (03.07.00)
Applicant UNIVERSITY OF MARYLAND, COLLEGE PARK et al	

1. The applicant is hereby notified that amendments to the claims under Article 19 were received by the International Bureau on:

08 January 2001 (08.01.01)

2. This date is within the time limit under Rule 46.1.

Consequently, the international publication of the international application will contain the amended claims according to Rule 48.2(f), (h) and (i).

3. The applicant is reminded that the international application (description, claims and drawings) may be amended during the international preliminary examination under Chapter II, according to Article 34, and in any case, before each of the designated Offices, according to Article 28 and Rule 52, or before each of the elected Offices, according to Article 41 and Rule 78.

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland Facsimile No.: (41-22) 740.14.35	Authorised officer Athina Nickitas-Etienne Telephone No.: (41-22) 338.83.38
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PATENT COOPERATION TREATY

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INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

REC'D 30 OCT 2001

WIPO

PCT

Applicant's or agent's file reference 55859-015	FOR FURTHER ACTION See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)	
International application No. PCT/US00/18325	International filing date (day/month/year) 03 JULY 2000	Priority date (day/month/year) 01 JULY 1999
International Patent Classification (IPC) or national classification and IPC Please See Supplemental Sheet.		
Applicant UNIVERSITY OF MARYLAND COLLEGE PARK		

- This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.
- This REPORT consists of a total of 6 sheets.
☐ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority. (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consist of a total of _____ sheets.

- This report contains indications relating to the following items:

- ☒ Basis of the report
- ☐ Priority
- ☐ Non-establishment of report with regard to novelty, inventive step or industrial applicability
- ☐ Lack of unity of invention
- ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- ☐ Certain documents cited
- ☒ Certain defects in the international application
- ☒ Certain observations on the international application

Date of submission of the demand 31 JANUARY 2001	Date of completion of this report 21 SEPTEMBER 2001
Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer JEFFREY E. RUSSEL Telephone No. (703) 308-0196

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/US00/18325

I. Basis of the report

1. With regard to the elements of the international application: *

☐ the international application as originally filed☒ the description:pages 1-37 , as originally filedpages NONE , filed with the demandpages NONE , filed with the letter of _____☒ the claims:pages 38-42 , as originally filedpages 42a , as amended (together with any statement) under Article 19pages NONE , filed with the demandpages NONE , filed with the letter of _____☒ the drawings:pages 1-8 , as originally filedpages NONE , filed with the demandpages NONE , filed with the letter of _____☒ the sequence listing part of the description:pages NONE , as originally filedpages NONE , filed with the demandpages NONE , filed with the letter of _____2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.
These elements were available or furnished to this Authority in the following language _____ which is:☐ the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).☐ the language of publication of the international application (under Rule 48.3(b)).☐ the language of the translation furnished for the purposes of international preliminary examination (under Rules 55.2 and/or 55.3).3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:☐ contained in the international application in printed form.☐ filed together with the international application in computer readable form.☐ furnished subsequently to this Authority in written form.☐ furnished subsequently to this Authority in computer readable form.☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.4. ☒ The amendments have resulted in the cancellation of:☒ the description, pages NONE☒ the claims, Nos. NONE☒ the drawings, sheets/fig NONE5. ☐ This report has been drawn as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).**

* Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17).

**Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/US00/18325

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability, citations and explanations supporting such statement

1. statement

Novelty (N)

Claims 4, 7-10, 14-20, 22, 24, and 25

YES

Claims 1-3, 5, 6, 11-13, 21, and 23

NO

Inventive Step (IS)

Claims 4, 7-10, 14-20, 22, 24, and 25

YES

Claims 1-3, 5, 6, 11-13, 21, and 23

NO

Industrial Applicability (IA)

Claims 1-25

YES

Claims NONE

NO

2. citations and explanations (Rule 70.7)

Claims 1-3, 5, 6, 11-13, 21, and 23 lack novelty under PCT Article 33(2) as being anticipated by Schally et al. Schally et al teach LHRH analogs, in which the second amino acid residue (counting from the N-terminus) is histidine, conjugated to nickel complexes. The complexes comprise salicylamine groups, cationic groups, and detectable labels. See, e.g., column 4, lines 3-4; column 8, lines 42-45, 48-49, and 61-63; and column 27, lines 64-68. The LHRH analogs of Schally et al correspond to Applicant's detectable protein ligands/labels. The conjugates of Schally et al are both functional derivatives of Applicant's Structures I, II, A, B, and C (all are capable of complexing nickel) and structural derivatives of Applicant's Structures I, II, A, B, and C (all comprise phenyl groups, amide groups, amine groups, etc.). Note that neither the claims nor the disclosure requires any particular degree of similarity between a derivative and Applicant's Structures I, II, A, B, and C.

Claims 1-3, 5, 11, 12, 21, and 23 lack novelty under PCT Article 33(2) as being anticipated by Mauclaire et al. Mauclaire et al teach compound (IV) which has Applicant's structure II. The compound can complex nickel and can be conjugated to an antigen, an antibody, or a hapten. See, e.g., column 5, lines 1-27, and column 8, lines 51-53 and 64-68. The antigens, antibodies, and haptens of Mauclaire et al correspond to Applicant's detectable labels. The conjugates of Mauclaire et al are both functional derivatives of Applicant's Structures I, II, A, B, and C (all are capable of complexing nickel) and structural derivatives of Applicant's Structures I, II, A, B, and C (all comprise phenyl groups, amine groups, pyridyl groups, etc.). Note that neither the claims nor the disclosure requires any particular degree of similarity between a derivative and Applicant's Structures I, II, A, B, and C.

Claims 1-3, 5, 6, 11, 12, 21, and 23 lack novelty under PCT Article 33(2) as being anticipated by Burrows et al. Burrows et al teach compounds 9 and 12 which have Applicant's structures I, II, A, and C. The compounds can complex nickel, and can be conjugated to proteins or protein fragments. See, e.g., column (Continued on Supplemental Sheet.)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/US00/18325

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:

Claims 1-15 and 17-25 are objected to under PCT Rule 66.2(a)(iii) as containing the following defect(s) in the form or contents thereof: At claim 1, page 39, line 4, "or" should be inserted before "oxygen". Claims 2 and 21 do not end with periods.

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

Claims 1-25 are objected to under PCT Rule 66.2(a)(v) as lacking clarity under PCT Article 6 because the claims are indefinite for the following reason(s): Claim 1 states that the compound comprises a labeled nickel complex "and" derivatives of Structures I-II. The use of the conjunction "and" indicates that the complexes and the structures are two distinct components of the compound. However, the structures require complexed nickel, and thus it is unclear if the complexes and the structures are actually distinct components. For analogous reasons, claim 21 is also indefinite. In claim 1, the term "derivatives" at claim 1, line 1, is indefinite because it is not clear what kind of similarity, e.g., structural and/or functional, and what degree of similarity, is necessary between a compound and Applicant's Structures I-II in order for the compound to be considered a derivative of Structures I-II and therefore to be embraced within the scope of Applicant's claims. The term is not defined either in Applicant's disclosure or in the art. For analogous reasons, the term "derivatives" in claims 2 and 21 is also indefinite. At claim 1, page 39, line 14, the phrase "continued polypeptide chain" is indefinite because it is not clear from what the polypeptide chain is to be continued. At claim 1, page 39, line 16, the phrase "simple amide" is indefinite because it is not clear what distinguishes a simple amide from a non-simple or complex amide. At claim 1, page 39, lines 16-18, the requirement for a "stable structure" is indefinite because "stable" is not defined either in Applicant's disclosure or in the art. Stability is a relative term and is highly dependent upon the environment in which the compound is located, but Applicant has provided no guidelines or conditions with which to determine whether or not a particular compound is stable or not. At claim 1, page 39, line 19, the "such as" phrase is indefinite because it is not clear if the variables are to be limited to the particularly recited variables or not. There is no antecedent basis in the claim for the phrases "the signal" and "the detectable label attached to the nucleic acid" in claims 16 and 22. Claim 17 refers to the "method according to claim 13"; however, claim 13 is directed to a compound, not a method. It is believed that claim 17 should instead depend upon claim 16. The preamble to claim 18 recites that a nucleic acid-containing adduct is to be purified; however, line 3 is limited to a solution of DNA. It is not clear if the claim is limited to purification of DNA, or if it embraces purification of nucleic acids generically. In claim 18, there is no antecedent basis in the claim for the phrases "the bound medium" (lines 4-5 indicate that an agent in the medium binds to the label) and "the solution mixture" (line 4 instead uses the terminology "the mixture"). Claim 25 is indefinite for the same reasons that claim 18 is. There is no antecedent basis in the claims for the phrase "the" (Continued on Supplemental Sheet.)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/US00/18325

Supplemental Box

(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Boxes I - VIII

Sheet 10

CLASSIFICATION:

The International Patent Classification (IPC) and/or the National classification are as listed below:

IPC(7): C07D 233/64, 235/02, 487/22; C07F 15/04; C07K 2/00, 14/795; G01N 33/52, 33/58, 33/60, 33/68 and US Cl.: 435/6; 436/86, 94; 530/300,400; 534/10, 12; 540/145; 548/106, 303.7; 556/32, 150

V. 2. REASONED STATEMENTS - CITATIONS AND EXPLANATIONS (Continued):

5, lines 60-67; column 6, lines 27-46; column 10, lines 34-39; and column 11, lines 4-39. The proteins and protein fragments of Burrows et al correspond to Applicant's detectable labels. The conjugates of Burrows et al are both functional derivatives of Applicant's Structures I, II, A, B, and C (all are capable of complexing nickel) and structural derivatives of Applicant's Structures I, II, A, B, and C (all comprise phenyl groups, amine groups, pyridyl groups, etc.). Note that neither the claims nor the disclosure requires any particular degree of similarity between a derivative and Applicant's Structures I, II, A, B, and C.

Claims 4, 7-10, 14-20, 22, 24, and 25 meet the criteria set out in PCT Article 33(2)-(3). With respect to instant claim 4, 14, and 15, the prior art does not teach or fairly suggest nickel complexes conjugated to biotin or to a flag element. With respect to instant claims 7-10, 16-20, 22, 24, and 25, the references discussed above do not teach or suggest Applicant's particular uses for their disclosed compounds.

Claims 1-25 meet the criteria set out in PCT Article 33(4). The claimed invention would have been expected to have industrial applicability in the detection and purification of nucleic acid sequences.

----- NEW CITATIONS -----

NONE

VIII. CERTAIN OBSERVATIONS ON THE APPLICATION (Continued):

detectable label" at claim 22, line 3. There is no antecedent basis in the claims for the phrase "the salicylamine containing nickel complex or nickel porphyrin complex" at claim 22, lines 3-4. Independent claim 21 does not use this terminology.

PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

To: JOSEPH H. KIM
MCDERMOTT, WILL & EMERY
600 13TH STREET, NW
WASHINGTON, DC 20005-3096

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL SEARCH REPORT OR THE DECLARATION

(PCT Rule 44.1)

Applicant's or agent's file reference 55859-015	Date of Mailing <i>(day/month/year)</i> <div style="font-size: 1.2em; margin-left: 100px;">14 NOV 2000</div>
International application No. PCT/US00/18325	International filing date <i>(day/month/year)</i> 03 JULY 2000
Applicant UNIVERSITY OF MARYLAND, COLLEGE PARK	

1. ☒ The applicant is hereby notified that the international search report has been established and is transmitted herewith.

Filing of amendments and statement under Article 19:

The applicant is entitled, if he so wishes, to amend the claims of the international application (see Rule 46):

When? The time limit for filing such amendments is normally 2 months from the date of transmittal of the international search report; however, for more details, see the notes on the accompanying sheet.

Where? Directly to the International Bureau of WIPO
 34, chemin des Colombettes
 1211 Geneva 20, Switzerland
 Facsimile No.: (41-22) 740.14.35

For more detailed instructions, see the notes on the accompanying sheet.

2. ☐ The applicant is hereby notified that no international search report will be established and that the declaration under Article 17(2)(a) to that effect is transmitted herewith.

3. ☐ With regard to the protest against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

☐ the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.

☐ no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Further action(s):** The applicant is reminded of the following:

Shortly after 18 months from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in rules 90 bis 1 and 90 bis 3, respectively, before the completion of the technical preparations for international publication.

Within 19 months from the priority date, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later).

Within 20 months from the priority date, the applicant must perform the prescribed acts for entry into the national phase before all designated Offices which have not been elected in the demand or in a later election within 19 months from the priority date or could not be elected because they are not bound by Chapter II.

Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer JEFFREY E. RUSSEL Telephone No. (703) 308-0196
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PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference 55859-015	<div style="display: flex; justify-content: space-between;"> <div style="text-align: center;">FOR FURTHER ACTION</div> <div>see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.</div> </div>	
International application No. PCT/US00/18325	International filing date (<i>day/month/year</i>) 03 JULY 2000	(Earliest) Priority Date (<i>day/month/year</i>) 01 JULY 1999
Applicant UNIVERSITY OF MARYLAND, COLLEGE PARK		

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 5 sheets.

☒ It is also accompanied by a copy of each prior art document cited in this report.

1. Basis of the report

a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.
☐ the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

2. ☐ **Certain claims were found unsearchable** (See Box I).

3. ☐ **Unity of invention is lacking** (See Box II).

4. With regard to the **title**,

- ☒ the text is approved as submitted by the applicant.
- ☐ the text has been established by this Authority to read as follows:

5. With regard to the **abstract**,

- ☐ the text is approved as submitted by the applicant.
- ☒ the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. 1

- ☒ as suggested by the applicant.
- ☐ because the applicant failed to suggest a figure.
- ☐ because this figure better characterizes the invention.
- ☐ None of the figures.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US00/18325

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : Please See Extra Sheet.

US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6; 436/86, 94; 530/300,400; 534/10, 12; 540/145; 548/106, 303.7; 556/32, 150

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WEST, CHEMICAL ABSTRACTS

search terms: Ni, nickel, porphyrin, salicylimine, salen, tmpy, nucleic, dna, rna, structures of claims 1 and 2

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,258,492 A (SCHALLY ET AL) 02 November 1993 (02/11/93), see column 4, lines 3-4, column 8, lines 42-45, 48-49, 61-63, column 27, lines 64-68.	1-3, 5, 6, 11, 12
X	US 5,268,371 A (MAUCLAIRE ET AL) 07 December 1993 (07/12/93), column 5, lines 1-27, column 8, lines 51-53, 64-68.	1-3, 5
X	US 5,272,056 A (BURROWS ET AL) 21 December 1993 (21/12/93), column 5, lines 60-67, column 6, lines 27-46, column 10, lines 34-39, column 11, lines 4-39.	1-3, 5, 6
A	US 5,595,726 A (MAGDA ET AL) 21 January 1997 (21/01/97).	1-20



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	* & * document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

12 OCTOBER 2000

Date of mailing of the international search report

14 NOV 2000

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
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Washington, D.C. 20231

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Authorized officer

JEFFREY E. RUSSEL

Telephone No. (703) 308-0196

Box III TEXT OF THE ABSTRACT (Continuation of item 5 of the first sheet)

The technical features mentioned in the abstract do not include a reference sign between parentheses (PCT Rule 8.1(d)).

NEW ABSTRACT

The invention relates to a compound comprising a labeled nickel complex (Figure 1) that is used to detect and purify nucleic acid sequences by forming an adduct between a specific nucleic acid base and the labeled nickel complex. The adduct can also be purified through a separation process, such as affinity chromatography.

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A,P	US 6,004,530 A (SAGNER ET AL) 21 December 1999 (21/12/99).	1-20
A,E	US 6,087,493 A (WHEELHOUSE ET AL) 11 July 2000 (11/07/00).	1-20
A	JP 3-38587 A (DOJINDO LABORATORIES) 19 February 1991 (19/02/91).	1-20
A	ROUTIER et al. Highly Preferential Cleavage Of Unpaired Guanines In DNA By A Functionalized Salen-Nickel Complex. Bioorganic & Medicinal Chemistry Letters. 1997, Volume 7, Number 1, pages 63-66.	1-20
A	WOODSON et al. A primer extension assay for modification of guanine by Ni(II) complexes. Nucleic Acid Research. 1993, Volume 21, Number 23, pages 5524-5525.	1-20

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/18325

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (7):

C07D 233/64, 235/02, 487/22; C07F 15/04; C07K 2/00, 14/795; G01N 33/52, 33/58, 33/60, 33/68

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/6; 436/86, 94; 530/300,400; 534/10, 12; 540/145; 548/106, 303.7; 556/32, 150

NOTES TO FORM PCT/ISA/220 (continued)

The following examples illustrate the manner in which amendments must be explained in the accompanying letter:

1. [Where originally there were 48 claims and after amendment of some claims there are 51]:
"Claims 1 to 29, 31, 32, 34, 35, 37 to 48 replaced by amended claims bearing the same numbers; claims 30, 33 and 36 unchanged; new claims 49 to 51 added."
2. [Where originally there were 15 claims and after amendment of all claims there are 11]:
"Claims 1 to 15 replaced by amended claims 1 to 11."
3. [Where originally there were 14 claims and the amendments consist in cancelling some claims and in adding new claims]:
"Claims 1 to 6 and 14 unchanged; claims 7 to 13 cancelled; new claims 15, 16 and 17 added." or
"Claims 7 to 13 cancelled; new claims 15, 16 and 17 added; all other claims unchanged."
4. [Where various kinds of amendments are made]:
"Claims 1-10 unchanged; claims 11 to 13, 18 and 19 cancelled; claims 14, 15 and 16 replaced by amended claim 14; claim 17 subdivided into amended claims 15, 16 and 17; new claims 20 and 21 added."

"Statement under Article 19(1)" (Rule 46.4)

The amendments may be accompanied by a statement explaining the amendments and indicating any impact that such amendments might have on the description and the drawings (which cannot be amended under Article 19(1)).

The statement will be published with the international application and the amended claims.

The statement should be brief, it should not exceed 500 words if in English or if translated into English.

It should not be confounded with and does not replace the letter indicating the differences between the claims as filed and as amended. It must be filed on a separate sheet and must be identified as such by a heading, preferably by using the words "Statement under Article 19(1)".

It should not contain any disparaging comments on the international search report or the relevance of citations contained in that report. Reference to citations, relevant to a given claim, contained in the international search report may be made only in connection with an amendment of that claim.

In what language?

The amendments must be made in the language in which the international application is published. The letter and any statement accompanying the amendments must be in the same language as the international application if that language is English or French; otherwise, it must be in English or French, at the choice of the applicant.

Consequence if a demand for international preliminary examination has already been filed?

If, at the time of filing any amendments under Article 19, a demand for international preliminary examination has already been submitted, the applicant must preferably, at the same time of filing the amendments with the International Bureau, also file a copy of such amendments with the International Preliminary Examining Authority (see Rule 62.2(a), first sentence).

Consequence with regard to translation of the international application for entry into the national phase?

The applicant's attention is drawn to the fact that, where upon entry into the national phase, a translation of the claims as amended under Article 19 may have to be furnished to the designated/elected Office, instead of, or in addition to, the translation of the claims as filed.

For further details on the requirements of each designated/elected Office, see Volume II of the PCT Applicant's Guide.

NOTES TO FORM PCT/ISA/220

These Notes are intended to give the basic instructions concerning the filing of amendments under Article 19. The Notes are based on the requirements of the Patent Cooperation Treaty and of the Regulations and the Administrative Instructions under that Treaty. In case of discrepancy between these Notes and those requirements, the latter are applicable. For more detailed information, see also the PCT Applicant's Guide, a publication of WIPO.

In these Notes, "Article", "Rule" and "Section" refer to the provisions of the PCT, the PCT Regulations and the PCT Administrative Instructions, respectively.

INSTRUCTIONS CONCERNING AMENDMENTS UNDER ARTICLE 19

The applicant has, after having received the international search report, one opportunity to amend the claims of the international application. It should however be emphasized that, since all parts of the international application (claims, description and drawings) may be amended during the international preliminary examination procedure, there is usually no need to file amendments of the claims under Article 19 except where, e.g. the applicant wants the latter to be published for the purpose of provisional protection or has another reason for amending the claims before international publication. Furthermore, it should be emphasized that provisional protection is available in some States only.

What parts of the international application may be amended?

The claims only.

The description and the drawings may only be amended during international preliminary examination under Chapter II.

When? Within 2 months from the date of transmittal of the international search report or 36 months from the priority date, whichever time limit expires later. It should be noted, however, that the amendments will be considered as having been received on time if they are received by the International Bureau after the expiration of the applicable time limit but before the completion of the technical preparations for international publication (Rule 46.1).

Where not to file the amendments?

The amendments may only be filed with the International Bureau and not with the receiving Office or the International Searching Authority (Rule 46.2).

When a demand for international preliminary examination has been filed, see below.

How? Either by cancelling one or more entire claims, by adding one or more new claims or by amending the text of one or more of the claims as filed.

A replacement sheet must be submitted for each sheet of the claims which, on account of an amendment or amendments, differs from the sheet originally filed.

All the claims appearing on a replacement sheet must be numbered in Arabic numerals. Where a claim is cancelled, no renumbering of the other claims is required. In all cases where claims are renumbered, they must be numbered consecutively (Administrative Instructions, Section 205(b)).

What documents may accompany the amendments?

Letter (Section 205(b)):

The amendments must be submitted with a letter.

The letter will not be published with the international application and the amended claims. It should not be confounded with the "Statement under Article 19(1)" (see below, under "Statement under Article 19(1)").

The letter must indicate the differences between the claims as filed and the claims as amended. It must, in particular, indicate, in connection with each claim appearing in the international application (it being understood that identical indications concerning several claims may be grouped), whether:

- (i) the claim is unchanged;
- (ii) the claim is cancelled;
- (iii) the claim is new;
- (iv) the claim replaces one or more claims as filed;
- (v) the claim is the result of the division of a claim as filed.

CORRECTED VERSION

(19) World Intellectual Property Organization
International Bureau(43) International Publication Date
11 January 2001 (11.01.2001)

PCT

(10) International Publication Number
WO 01/02370 A1(51) International Patent Classification⁷: C07D 233/64,
235/02, 487/22, C07F 15/04, C07K 2/00, 14/795, G01N
33/52, 33/58, 33/60, 33/68

(21) International Application Number: PCT/US00/18325

(22) International Filing Date: 3 July 2000 (03.07.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/141,965 1 July 1999 (01.07.1999) US(81) Designated States (*national*): AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.(84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).(71) Applicants (*for all designated States except US*): UNIVERSITY OF MARYLAND, COLLEGE PARK [US/US]; 6200 Baltimore Avenue, Riverdale, MD 20737 (US). UNIVERSITY OF UTAH [US/US]; Technology Transfer Office, Suite #110, 615 Arapleen Drive, Salt Lake City, UT 84108 (US).

(72) Inventors; and

(75) Inventors/Applicants (*for US only*): ROKITA, Steven, E. [US/US]; 1105 Brantford Avenue, Silver Spring, MD 20904 (US). BURROWS, Cynthia, J. [US/US]; 1333 Chandler Drive, Salt Lake City, UT 84103 (US).

(74) Agents: KIM, Joseph, H. et al.; McDermott, Will & Emery, 600 13th Street, NW, Washington, DC 20005-3096 (US).

Published:

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- With amended claims.

Date of publication of the amended claims: 3 May 2001

(48) Date of publication of this corrected version:

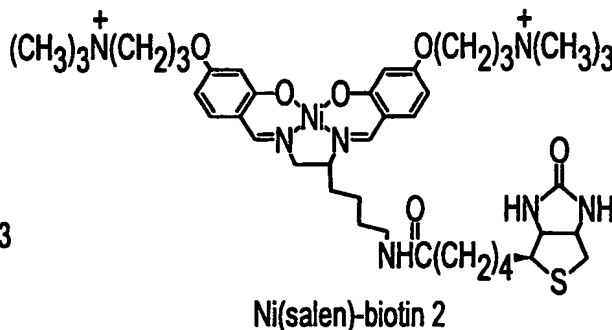
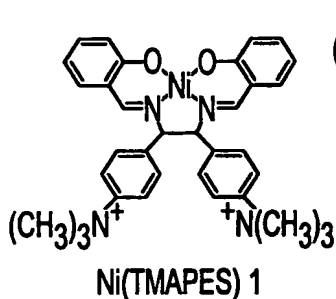
12 July 2001

(15) Information about Correction:

see PCT Gazette No. 28/2001 of 12 July 2001, Section II

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: NICKEL-BASED REAGENTS FOR DETECTING DNA AND DNA-PROTEIN CONTACTS



(57) Abstract: The invention relates to a compound comprising a labeled nickel complex (Figure 1) that is used to detect and purify nucleic acid sequences by forming an adduct between a specific nucleic acid base and the labeled nickel complex. The adduct can also be purified through a separation process, such as affinity chromatography.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US00/18325

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : Please See Extra Sheet.

US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6; 436/86, 94; 530/300,400; 534/10, 12; 540/145; 548/106, 303.7; 556/32, 150

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WEST, CHEMICAL ABSTRACTS

search terms: Ni, nickel, porphyrin, salicylimine, salen, tmpy, nucleic, dna, rna, structures of claims 1 and 2

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,258,492 A (SCHALLY ET AL) 02 November 1993 (02/11/93), see column 4, lines 3-4, column 8, lines 42-45, 48-49, 61-63, column 27, lines 64-68.	1-3, 5, 6, 11, 12
X	US 5,268,371 A (MAUCLAIRE ET AL) 07 December 1993 (07/12/93), column 5, lines 1-27, column 8, lines 51-53, 64-68.	1-3, 5
X	US 5,272,056 A (BURROWS ET AL) 21 December 1993 (21/12/93), column 5, lines 60-67, column 6, lines 27-46, column 10, lines 34-39, column 11, lines 4-39.	1-3, 5, 6
A	US 5,595,726 A (MAGDA ET AL) 21 January 1997 (21/01/97).	1-20

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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E earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

12 OCTOBER 2000

Date of mailing of the international search report

14 NOV 2000

Name and mailing address of the ISA/US
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Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

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Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US00/18325

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A,P	US 6,004,530 A (SAGNER ET AL) 21 December 1999 (21/12/99).	1-20
A,E	US 6,087,493 A (WHEELHOUSE ET AL) 11 July 2000 (11/07/00).	1-20
A	JP 3-38587 A (DOJINDO LABORATORIES) 19 February 1991 (19/02/91).	1-20
A	ROUTIER et al. Highly Preferential Cleavage Of Unpaired Guanines In DNA By A Functionalized Salen-Nickel Complex. Bioorganic & Medicinal Chemistry Letters. 1997, Volume 7, Number 1, pages 63-66.	1-20
A	WOODSON et al. A primer extension assay for modification of guanine by Ni(II) complexes. Nucleic Acid Research. 1993, Volume 21, Number 23, pages 5524-5525.	1-20

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/18325

A. CLASSIFICATION OF SUBJECT MATTER:

IPC (7):

C07D 233/64, 235/02, 487/22; C07F 15/04; C07K 2/00, 14/795; G01N 33/52, 33/58, 33/60, 33/68

A. CLASSIFICATION OF SUBJECT MATTER:

US CL :

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235/02, 487/22, C07F 15/04, C07K 2/00, 14/795, G01N
33/52, 33/58, 33/60, 33/68

(21) International Application Number: PCT/US00/18325

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(25) Filing Language: English

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(30) Priority Data:
60/141,965 1 July 1999 (01.07.1999) US

(71) Applicant (for all designated States except US): **UNIVERSITY OF MARYLAND, COLLEGE PARK** [US/US];
6200 Baltimore Avenue, Riverdale, MD 20737 (US).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **ROKITA, Steven, E.** [US/US]; 1105 Brantford Avenue, Silver Spring, MD 20904 (US). **BURROWS, Cynthia, J.** [US/US]; 1333 Chandler Drive, Salt Lake City, UT 84103 (US).

(74) Agents: **KIM, Joseph, H. et al.**; McDermott, Will & Emery, 600 13th Street, NW, Washington, DC 20005-3096 (US).

(81) Designated States (national): AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

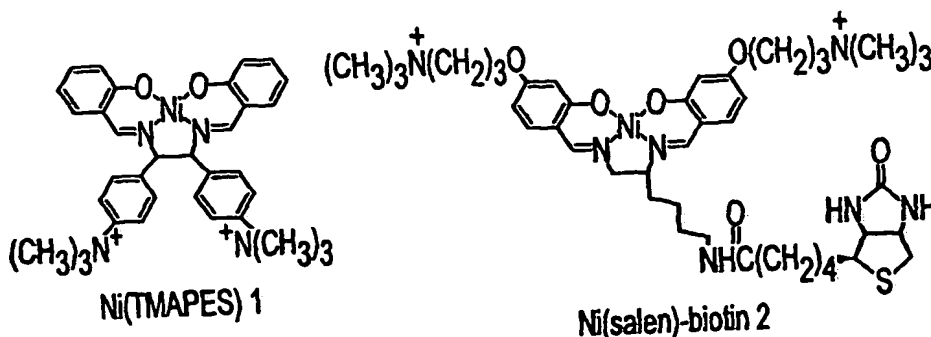
Published:

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- With amended claims.

Date of publication of the amended claims: 3 May 2001

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: NICKEL-BASED REAGENTS FOR DETECTING DNA AND DNA-PROTEIN CONTACTS



(57) Abstract: The invention relates to a compound comprising a labeled nickel complex (Figure 1) that is used to detect and purify nucleic acid sequences by forming an adduct between a specific nucleic acid base and the labeled nickel complex. The adduct can also be purified through a separation process, such as affinity chromatography.

WO 01/02370 A1

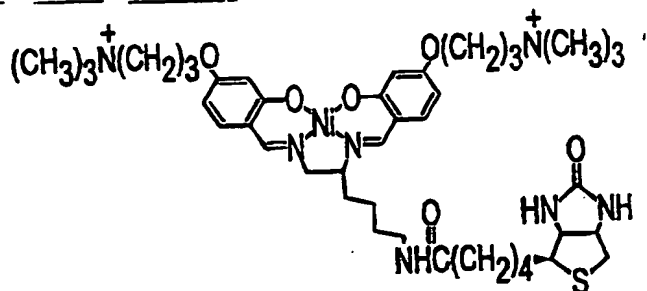
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02 JAN 2002

AMENDED CLAIMS

[received by the International Bureau on 8 January 2001 (08.01.01);
new claims 21-25 added; remaining claims unchanged (1 page)]

21. A compound comprising a labeled nickel complex and derivatives of



22. A method for detecting a non-canonical nucleic acid sequence comprising binding the compound according to claim 21 to a sample of nucleic acid, and detecting a signal of the detectable label on the salicylimine containing nickel complex or nickel porphyrin complex.

23. A labeled hybrid compound comprising the compound according to claim 21, complexed with a protein.

24. A method for detecting or measuring protein-nucleic acid interaction comprising mixing the compound according to claim 23 with a solution of nucleic acid, and assaying for the signal from the detectable label attached to the nucleic acid.

25. A method for purifying a nucleic acid-nickel-complex adduct, comprising:
mixing a labeled compound according to claim 21 with a solution of DNA,
subjecting the mixture to a separation medium, wherein the medium contains an agent that specifically binds to the label, and
separating the bound medium from the solution mixture, wherein the adduct is bound to the specific agent.

NICKEL-BASED REAGENTS FOR DETECTING DNA AND DNA-PROTEIN CONTACTS

CONTINUING DATA

This application claims the benefit of priority to U. S. Provisional Application No. 60/141,965, filed July 1, 1999, the contents of which are incorporated herein by reference in their entirety.

GOVERNMENT INTEREST IN THE INVENTION

This invention was made with Government support under Grant Nos. GM-47531 and GM-49860 awarded by the National Institutes of Health. The Government has certain rights in this invention.

BACKGROUND OF THE INVENTION

The present invention relates to a nickel-based reagent that covalently binds to a specific nucleotide. The present invention also relates to a nickel-based salicylimine complexed to a histidine containing organic compound, as well as a nickel-based complex comprising a detectable label.

There are a variety of methods for detecting DNA/RNA-protein interactions and some depend on cross-linking. However, to the inventors' knowledge, there is no current technology that uses a nickel-based complex to covalently bind to a specific nucleotide and detect such binding and optionally isolating the DNA regions through use of a labeled complex.

A salicylimine containing nickel complex or any square planar, 4-coordinate nickel complex system linked with any detectable label is sufficient to practice the present invention. Moreover, the salicylimine containing nickel group complexed with the N-terminus of a polypeptide where the terminal amino acid is followed by a histidine or histidiny residue results in a nickel based reagent that has additional desirable functional

capabilities. If this terminal amino acid sequence is not naturally derived, then genetic manipulation may be used to append such a terminus on to any polypeptide of interest. For non-natural amino acids, chemical synthesis is preferred. Further, a semi-synthesis approach can be taken. Combining genetic and chemical means to obtain an amino acid sequence complexed with the salicylimine containing nickel complex is encompassed by the present invention. In addition, this complex may be used to add an aromatic aldehyde selectively to a nucleotide sequence which can be used as a handle for further modification or identification. Furthermore, whereas single-stranded regions of nucleic acids typically react more readily with various chemical and biochemical reagents, their detection and characterization method require prior knowledge of the target sequence. A nickel complex-identifier (such as biotin) of the invention will allow for purification and identification of the nucleic acid sequence without prior knowledge of the reactive sequence. Whereas there is no current reagent that selectively couples to and stays attached to the bound nucleotide, the nickel reagent of the invention allows for the detection of the bound sequence as well as isolation of the nucleotide region.

Shearer, J. M. and Rokita, S.E., *Bioorg. Med. Chem. Lett.* 9, 501-504, (1999), discloses the preparation of diamine compound for synthesis of a water-soluble Ni (II)-salen (salicylaldehyde) complex. This reference discloses a synthesis method for a simple, unconjugated Ni (Salen) called TMAPES. However, this reference does not disclose the attachment of a label or an adduct to this complex so that the complex can be detected biochemically.

Liang et al., *J. Am. Chem. Soc.*, 120, 248-257 (1998), discloses a Ni (II) linked metalloprotein that is used to selectively degrade DNA through a minor groove binding interaction. However, this reference does not disclose or suggest the covalent linkage between a nickel containing complex and a specific nucleotide.

Routier et al., *Bioconjugate Chem.*, 8, 789-792 (1997), discloses a conjugate between a salen and any biologically active molecule. The complex uses copper. However, such a copper conjugate cleaves nucleic acids non-specifically while non-covalently binding to the backbone of the nucleic acid. This is contrary to the present invention, which is directed to specific coupling of the nickel-containing complex to a

nucleotide base.

Routier et al., *Bioorganic & Medicinal Chemistry Letters*, Vol. 7, No. 1, pp. 63-66 (1997), discloses a Ni-Salen complex. However, in this reference, coupling the Ni-Salen complex with the terminal N group would typically create a neutral compound that would be insoluble and not useful in coupling to nucleic acid bases.

Bhattacharya and Mandal, *J. Chem. Soc., Chem. Commun.*, 1995, pp. 2489-2490, discloses a Co-Salen chemistry. Again, this reference does not disclose formation of an adduct with nucleic acid.

Tanaka et al., *Bull. Chem. Soc., Jpn.* 70, 615-629 (1997), discloses the synthesis of a Cu-Salen molecule. This reference does not disclose or suggest formation of an adduct with nucleic acids.

Gravert and Griffin, *J. Org. Chem.*, 58, 820-822 (1993), discloses a Mn-Salen complex. This reference does not disclose the formation of an adduct with nucleic acids.

Chen and Lu, *Journal of the Chinese Chemical Society*, 45, 611-617 (1998), discloses a Ru-Salen complex. This reference does not disclose or suggest the formation of an adduct with nucleic acids.

Jacquet et al., *J. Am. Chem. Soc.* 119, 11763-11768, (1997), discloses a Ru metal - Salen complex which requires photo-activation.

Gravert and Griffin, *Inorg. Chem.*, 35, 4837-4847 (1996), discloses a Mn-Salen complex. However, this reference does not disclose the formation of an adduct with nucleic acids.

Brown et al., *Biochemistry*, 34, 4733-4739 (1995), discloses a nickel-His-tag and a nickel-glycylglycylhistidine for protein-protein cross-linking. However, the reference does not disclose nickel-dependent protein-nucleic acids or nucleic acid-biotin cross-linking.

U. S. Patent Nos. 5,272,056 and 5,504,075 disclose a Ni(TMAPES) complex but fails to disclose attaching a detectable label to this complex. These two patents are incorporated herein by reference in their entirety.

As the literature described above shows, no salicylimine containing nickel complex is known in the prior art for the application of directly detecting or isolating nucleic acids. There is a need in the art for a more effective method of studying chromosome structure,

detecting specific nucleotide bases, and studying protein-DNA interactions.

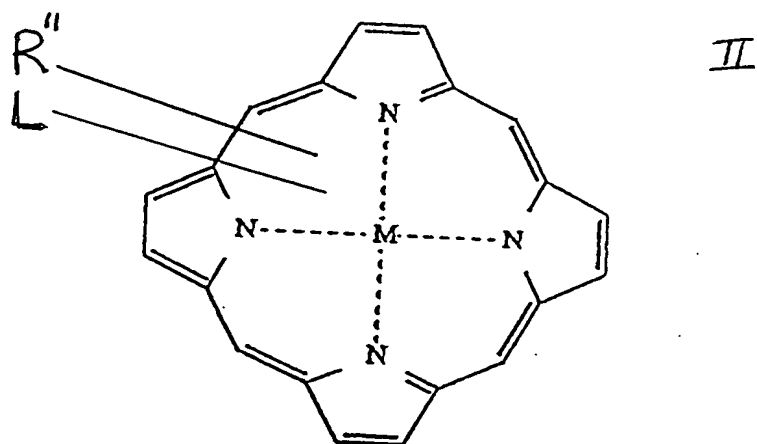
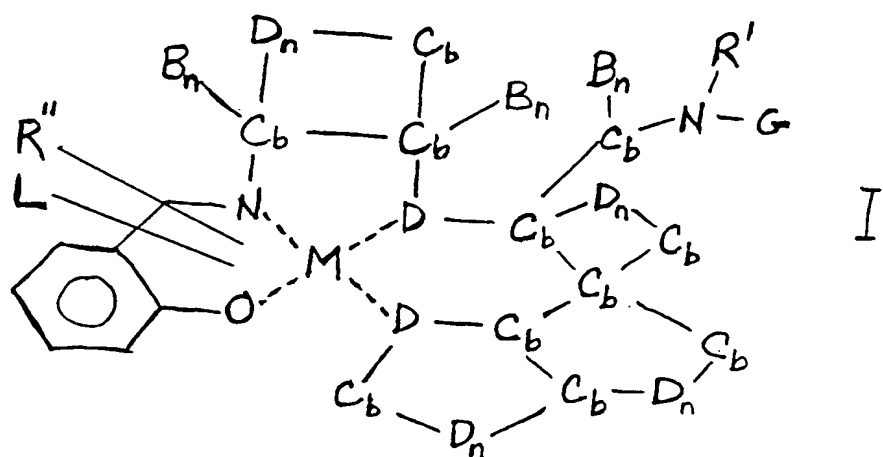
The invention provides a salicylimine containing nickel complex for detecting protein-nucleic acid interactions and the presence of single-stranded regions of nucleic acids formed during gene expression, macromolecular assembly and chromatin reorganization.

SUMMARY OF THE INVENTION

The present invention has met all of the needs stated above.

Accordingly, there is provided a process for oxidatively modifying a nucleic acid comprising the steps of providing a nucleic acid containing a target nucleotide which includes guanine, and providing a nickel-reagent that has a square planar, 4-coordinate system linked to a detectable label, which is capable of oxidative covalent coupling to the nucleic acid at or near the target sequence in the presence of an oxidizing agent. Preferably, the nickel-reagent is a salicylimine containing nickel complex, or a nickel-porphyrin complex. The nucleic acid is contacted with the salicylimine containing metal complex in the presence of an oxidizing agent so that the nucleic acid is modified at or near the target nucleotide. This also allows for the isolation of the bound nucleotide sequence region.

Structures for the nickel complex are derivatives of Structures I-II:



wherein:

B independently represents doubly bonded oxygen;

C represents carbon;

D independently represents nitrogen, oxygen;

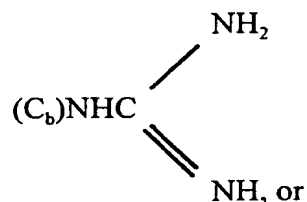
L is a detectable label, optionally attached to a linker;

M represents a nickel ion;

b=0-6;

n=0-1;

R independently represents a cationic group, optionally attached to a linker, wherein said cationic group is at least one C_b group linked to a nitrogen atom, $(CH_2)_3NH_2$, $(CH_2)_4NH_2$, $C_bN(C_b)_{0-3}$,



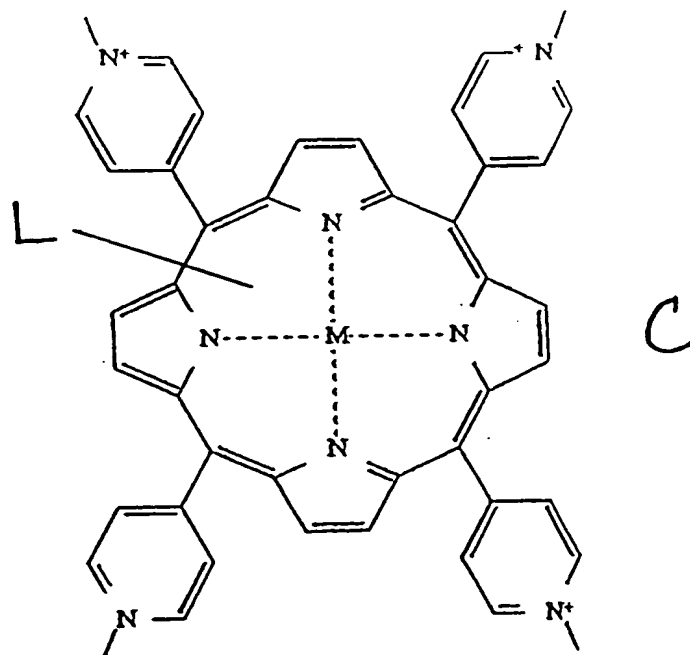
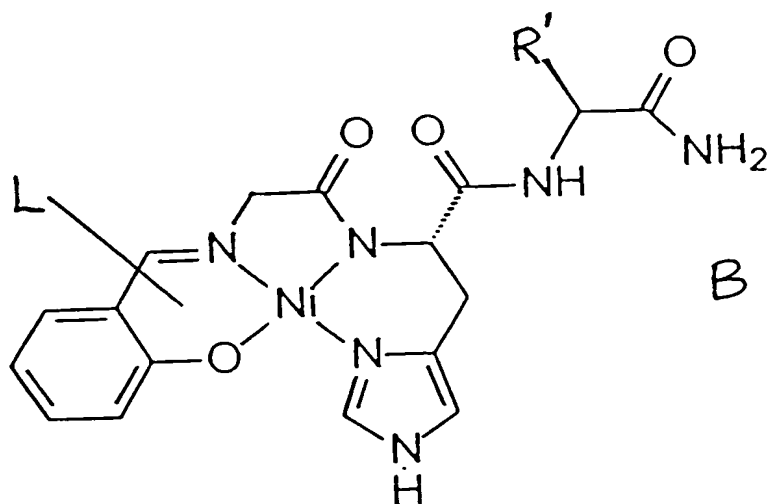
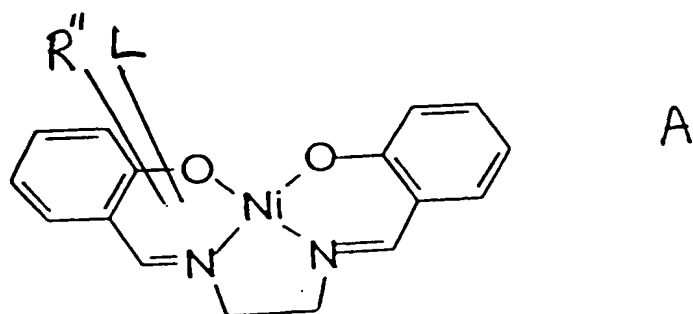
pyridyl;

R' represents hydrogen, alkyl, aryl or a continued polypeptide chain;

R'' is R or R' or G;

G represents OH, OR, a simple amide or a DNA delivery agent; and wherein all atoms contain sufficient bonds to adjacent atoms, to other atoms or to hydrogen to result in a stable structure, wherein, by independently representing is meant that within one structure, all values for the variables such as B, C, D, G, L, M, R, R', R'', b, n, need not be the same, but may represent different atoms or numbers within a single structure.

The nickel complex of Structures I-II may be structures A-C or derivatives thereof:



A kit is also provided for a nucleic acid assay for locating guanine groups in nucleic acids, oligonucleotides, polynucleotides, RNA and single-stranded DNA, and non-base paired guanine groups in double-stranded DNA. The kit comprises a labeled salicylimine containing metal complex selected from Structures I-II and their derivatives, an oxidant selected from the group consisting of peracid, sulfite and O₂, and optionally a base of piperidine, N-butylamine or sodium hydroxide when scission is desired.

Advantageously, the process of the invention provides a new method for detecting and isolating non-canonical DNA regions through guanine-specific modification of DNA.

Another object of the invention is to provide a nickel-based reagent to which is attached a detectable label so that the binding of the nickel-based reagent to a specific nucleotide base is detected by assaying for the label. The nucleic acid base is typically guanosine. A practical effect of the ability to detect the binding of the nickel-based reagent to the nucleic acid is the ability to isolate the bound DNA region.

It is another object of the present invention to provide a nickel-based reagent complex that is complexed with an amino acid sequence. Preferably, the amino acid sequence includes histidine. The label can be attached to either the Salen complex itself or to one of the amino acids. Preferably, Salen is complexed with an α -amino group on the N-terminal end of the amino acid sequence, and a histidine group, so that a square planar complex is formed with a nickel ion complexed with it.

Another object of the invention is to provide a reagent kit to serve industrial and academic research laboratories in research related to discerning the chromatin structure and protein-nucleic acid interaction.

These and other objects of the invention will be more fully understood from the following description of the invention, the referenced drawings attached hereto and the claims appended hereto.

BRIEF DESCRIPTION OF THE DRAWINGS

The present invention will become more fully understood from the detailed description given hereinbelow, and the accompanying drawings which are given by way of illustration only, and thus are not limitative of the present invention, and wherein;

FIGURE 1 shows Ni(salen) probes for nucleic acid structure.

FIGURE 2 shows synthesis of a Ni(salen)-biotin conjugate with solubility in water.

Reagents and conditions (a), Et_3N , ClCOOEt , NH_3 , THF, 10°C -rt, 56 %. (b) $(\text{CF}_3\text{CO})_2\text{O}$, Et_3N , THF, N_2 , 0°C , 87 %. (c) H_2 , Ra-Ni, 6.8 atm, NH_3 , MeOH, 3 days, 72 %. (d) H_2 , Pd/C, MeOH, rt, overnight, 82 %. (e) 2, 4-dihydroxy-benzaldehyde, NiAc_2 , EtOH, reflux, N_2 , 3hr, 78 %. (f) TFA, CH_2Cl_2 , rt, 0.5 h, 86 %. (g) BNHS, Et_3N , DMF, 4°C , 3 days, 22 %. (h) $\text{Br}(\text{CH}_2)_3\text{N}(\text{CH}_3)_3\text{Br}$, DMF, rt, 3days, 80 %.

FIGURE 3 shows a phosphorimage of a denaturing polyacrylamide gel (20%, 7 M urea) used to identify the high-molecular weight conjugate formed between DNA and Ni(salen)-biotin. The 5'- ^{32}P labeled oligodeoxynucleotide (18' mer), 5'-d(AAAATATCAGATCTAAAA) (12 mM, 6 nCi), in 10 mM sodium phosphate pH 7 (lane 1) was alternatively incubated with Ni(salen)-biotin 2 (50 mM, lane 2), MMPP (120 mM, lane 3) and their combination (lane 4). The resulting DNA conjugate was isolated from a monomeric avidin affinity column (lane 5) and subsequently treated with piperidine (0.2 M, 90°C , 30 min, lane 6). A standard G-lane was generated by dimethylsulfate as described by Maxam and Gilbert (lane 7) (Maxam, A. M.; Gilbert, W. *Methods Enzymol.* **1980**, 65, 499-560). As a control, the parent oligonucleotide was also treated with piperidine (0.2 M, 90°C , 30 min, lane 8).

FIGURE 4 shows the formula for $(\text{Ni}(\text{salen-ArgHis}))^+$.

FIGURE 5 shows an ORTEP plot of $[\text{Ni}(\text{salen-ArgHis})]\text{CH}_3\text{CO}_2 \cdot 3\text{H}_2\text{O}$, **8**; the hydrogen atoms are omitted for clarity. Selected bond lengths (\AA) and angles (deg): Ni-O(1), 1.8555(14); Ni-N(1), 1.8576(14); Ni-N(2), 1.8721(15); Ni-N(3), 1.8931(14); N(1)-C(7), 1.285(3); N(1)-C(8), 1.484(2); N(2)-C(9), 1.337(2); N(2)-C(10), 1.459(2); N(5)-C(15), 1.327(3); O(1)-Ni-N(1), 94.05(7); O(1)-Ni-N(2), 177.08(8); N(1)-Ni-N(2), 85.14(7); O(1)-Ni-N(3), 87.91(7); N(1)-Ni-N(3), 177.54(7); N(2)-Ni-N(3), 92.98(7).

FIGURE 6 shows phosphorimage of 20% polyacrylamide denaturing gel (7 M urea) showing high-molecular weight adducts to DNA formed with nickel complexes. The 12-mer oligodeoxynucleotide, 5'-d(ATATCAGATCTA)-3', was labeled at the 5' terminus with ^{32}P and incubated with reactants under the standard conditions described in Supporting Information. Lane 1: DNA alone. Lane 2: control with 100 μM Ni(salen- ArgHis) only. Lanes 3-6: 100 μM 8 plus the indicated concentrations of oxidant. Lane 7: control showing the effect of Na_2SO_3 without metal complex. Lane 8: control with 2 μM 9. Lane 9: 2 μM 9 plus Na_2SO_3 . All experiments were carried out in the presence of air and included EDTA in the workup. Cross-linking yields in lanes 3, 4, 5, and 6 were 16, 36, 39, and 80%, respectively.

FIGURE 7 shows the effect of piperidine treatment of the DNA-Ni(salen-ArgHis) adduct. Reactions were run in a similar manner as those in FIGURE 6. Lane 1: control using 100 μM Ni(OAc) $_2$, 100 μM ArgHis dipeptide and 1 mM Na_2SO_3 , showing that a high-molecular-weight species does not form in the absence of salicylaldehyde. Lanes 2-6: Reactions with 100 μM 1 and 1mM Na_2SO_3 followed by piperidine treatment (0.2M, 90°C, 30 min) when so indicated. Lanes 2 and 3: 12-mer oligonucleotide band cut and isolated from a previous gel after the adduct had formed. Lanes 4 and 5: Band corresponding to high-molecular-weight adduct cut and isolated from a previous gel. Lane 6: Typical reaction of 100 μM 1, 1 mM Na_2SO_3 with piperidine treatment. Lane G: Maxam-Gilbert G lane using dimethylsulfate.

FIGURE 8 shows ESI-MS spectrum of the high molecular weight adduct isolated from the reaction of the 12-mer oligodeoxynucleotide and Ni(salen-ArgHis) with sulfite and O_2 . The molecular weight of the 12-mer itself is 3628 as determined by ESI-MS. (R.J. Perse, Ph.D., Dissertation, University of Utah, 1997) The proposed structure of the salicylaldehyde adduct is shown.

FIGURE 9 shows a schematic for synthesizing Ni(salen-Lys(biotin)HisArg) 12.

DETAILED DESCRIPTION OF THE INVENTION

The inventors have developed a series of labeled nickel-based complex that are square planar and 4-coordinate systems. Salicylimine containing nickel-based complexes to detect protein-DNA (RNA) interactions have been exemplified in the instant application. One of the advantages of the invention is that by using the complex of the present invention, regulatory regions of DNA can be identified and characterized. Furthermore, chromatin structure abnormalities can also be examined, and epigenetic modifications of chromosomes can be determined.

In a preferred embodiment, a model peptide-salicylaldehyde complex was shown to exhibit the predicted ability to cross-link to DNA in the presence of nickel and an oxidant such as sulfite. This cross-link can either be maintained or further modified to leave only an aromatic aldehyde attached to the site of modification for additional conjugation to standard detection and purification appendages. This technology is now extended to full proteins and may also be elaborated for detection through standard protocols involving biotin, digoxigenin or a flag sequence recognition molecule, such as green fluorescent protein or a specific epitope. Furthermore, a related salicylaldehyde complex has been linked to biotin for detecting single-stranded regions of DNA. This compound has been synthesized and has been tested with DNA.

It is to be understood that the invention is not limited to the particular nickel complex described above. At a minimum, the complex requires that at least the complex take a square planar, 4-coordinate system. Preferably, the complex may contain a salicylimine containing component, preferably salicylaldehyde, and one or more additional component serving as a ligand to generate a square planar complex with nickel. In addition to the salicylimine containing group, the nickel complex may be complexed with porphyrin to provide a square-planar, 4-coordinate system. Additional components could be formed by linking other natural or non-natural organic compounds such as amino acids to the complex. The complex should also be water-soluble, by for example, the presence of at least one positive charge on the complex in water. In addition, the complex can be

conjugated to recognize elements to facilitate cellular or biomolecular localization in addition to purification and identification again through standard protocols involving biotin, flag sequence, antibodies and so on.

Abbreviations

Rink Amide AM resin	4-(2',4'-dimethoxyphenyl-Fmoc-aminomethyl-phenoxyacetamido-norleucylaminomethyl resin
Fmoc	Fluorenylmethoxycarbonyl
Pbf	2,2,4,6,7-pentmethyldihydrobenzofuran-5-sulfonyl
Trt	trityl
Dde	1-(4,4-dimethyl-2,6-dioxo-cyclohexylidene)ethyl
HBTU	2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
DMF	dimethylformamide
TIS	triisopropylsilane
TFA	trifluoroacetic acid

Abbreviations for amino acids used herein are conventionally defined as described hereinbelow unless otherwise indicated.

<u>Amino Acid</u>	<u>Three-letter abbreviation</u>	<u>One-letter symbol</u>
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Asparagine or aspartic acid	Asx	B
Cysteine	Cys	C
Diaminopropionic acid	Dpr	

Glutamine	Gln	Q
Glutamine acid	Glu	E
Glutamine or glutamic acid	Glx	Z
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Ornithine	Om	
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V
Unknown	Xaa	X

Salicylimine Containing Compounds

The compounds useful in the invention are characterized in one regard by their ability to form stable complexes with transition metals. Stable complexes are those having measurable lifetimes at room temperature in water or common organic solvents.

The compounds comprise at least one ligand and a transition metal. A ligand is defined herein as a molecule that is attached to the central metal atom of a coordination

compound. Preferred ligands are tetradentate or pentadentate and may comprise either macrocyclic or non-macrocyclic molecules. Other ligands, however, can also be used. It is to be understood that a tetradentate ligand has four donor atoms while a pentadentate ligand has five donor atoms.

The donor atoms may be nitrogen or oxygen. Preferably, at least two donor atoms are nitrogens, which are separated by from two to seven carbon atoms.

Suitable nitrogen donor groups are the amino group of peptides, or amines, imines, pyridines, imidazoles, pyrroles, and pyrazoles, with imine and pyridine groups preferred. The configuration may be square planar or pyramidal, with square planar preferred, but is not limited to these. Suitable oxygen donor groups are phenol, alcohol, carboxylic acid, and carbonyl. Examples of molecules containing oxygen donor groups are salens and salophens.

The ability of the ligands to form stable complexes results from the relative positions of the donor groups. Much of the rest of the ligand consists of carbon atoms that may be thought of as collectively forming a scaffold for maintaining the proper position of the donor groups.

Substituents on the atoms of the ligand affect the properties of the compounds, such as their ability to bind the nucleotides and DNA, their effectiveness in participating in the modification of nucleotides and DNA, their solubility in various solvents, and the stability of the complex they form with transition metals. The atoms of the ligands are normally substituted with sufficient hydrogen atoms to form a stable compound. It should be appreciated, however, that any positions in the ligands, whether or not so indicated herein, may be substituted with any other group and still do substantially the same thing in substantially the same way to accomplish the same result and are, therefore, to be considered equivalent to positions bearing hydrogen atoms as substituents for the purpose of determining the scope of the present invention.

Other suitable ligands are porphyrins such as structure C.

Some of the positions shown in Structures I-II and Structures A-C do not appear to be substituents other than hydrogen. Nevertheless, these positions may be substituted by any organic or inorganic group without significantly affecting the ability of the compound

to form a complex with transition metals.

Accordingly, any one or more of these positions may be substituted by an inorganic substituent, such as a doubly bonded oxygen, i.e., carbonyl, or a singly bonded oxygen i.e., hydroxy. Some additional inorganic groups include, for example, amino, thio, halo, i.e., F, Cl, Br, and I, etc.

Organic substituents include, for example, alkyl, aryl, alkylaryl and arylalkyl. The alkyl groups may be branched or unbranched and contain 20 carbon atoms or less, preferably 8 carbon atoms or less, and more preferably 4 carbon atoms or less. Some typical examples of alkyl groups include methyl, ethyl, n-propyl, isopropyl, n-butyl, sec-butyl, tert-butyl, isobutyl, and octyl. The alkyl groups may, in whole or in part, be in the form of rings such as cyclopentyl, cyclohexyl, cycloheptyl and cyclohexylmethyl. The cyclic groups described above may be further substituted with inorganic, alkyl, or aryl groups. Any of the alkyl groups described above may have one or more double or triple bond. Moreover, any of the carbon atoms of the alkyl groups may be separated from each other or from the ring nucleus with groups such as carbonyl, oxycarbonyl, oxy, amino, thio, etc. Alkyl groups may also terminate with groups such as halo, hydroxy, amino, carboxy, etc.

Aryl substituents are typically phenyl, but may also be any other aryl groups such as, for example, pyrrolyl, furanyl, pyridyl, thiazolyl, etc. The aryl group may, further, be substituted by an inorganic, alkyl, or other aryl group. The alkylaryl and arylalkyl groups may be any combination of alkyl and aryl groups. These groups may be further substituted.

Particularly important substituent groups which may be present on the ligands are appended groups which are capable of binding to DNA or nucleotides. These will be called delivery agents. Suitable delivery agents may be of different types including intercalators, groove-binding agents, oligonucleotides, proteins or protein fragments, and polyamines. The intercalators include ethidium, methidium, acridine, proflavin, phenanthroline, etc. The groove-binding agents include distamycin and netropsin. A distamycin derivative was used to direct nucleotide scission by Schultz, P. G., et al., "Design and Synthesis of a Sequence-Specific DNA Cleaving Molecule. (Distamycin-EDTA) iron(II)", J. Am. Chem. Soc. 104, 6861-6863 (1982). Oligonucleotides may bind producing double helical or triple

helical areas. Proteins or protein fragments which bind DNA include Hin recombinase (Mack, D. P. and Dervan, P. B., "Nickel-Mediated Sequence-Specific Oxidative Cleavage of DNA by a Designed Metalloprotein", J. Am. Chem. Soc. 112, 4604-4606 (1990)). The useful polyamines include spermine and spermidine described by K. D. Stewart, "The Effect of Structural Changes in a Polyamine Backbone on its DNA-Binding Properties", Biochem, Biophys. Res. Comm. 152, 1441-1446 (1988).

The Labeled Complexes

Ligands to be used in the invention form stable complexes with transition metal ions. For the purpose of this specification, transition metals are to be understood as including metals having partly filled d or f shells in any of their commonly occurring oxidation states. The useful transition metals include ions of Ni, Co, Cu, Rh, Pd, Ir, Pt, Cr, Mn, Fe, Ru, and Os. Preferred are ions of Ni, Co and Pd. Most preferred is Ni.

The complexes are formed by contacting a salt of the metal ion with the ligand in a suitable solvent, for example, water and methanol. Progress of the complexation is easily followed visually or spectrophotometrically. Depending upon reaction conditions such as the ligand, the metal, the pH, and the solvent, the complexation reaction may occur rapidly at room temperature, or may require heating.

The complexes may be four, five or six coordinate with four preferred and square planar most preferred.

The complexes may be deprotonated, but an overall positive charge is preferred, and a highly positive charge is most preferred. Balancing anions, counterions, or salts may be as known to those skilled in the art, such as derivatives of salts e.g. perchlorate, acetate, nitrate, chlorides, bromides, iodides, and tetrachlorozincate (ZnCl_4^{-2}).

The ligand-metal complexes are used in the process of the invention to modify oligonucleotides, single-stranded DNA, non-classical duplex structures and double-stranded DNA having particular conformations, that is, extra-helical areas, cruciform DNA, abasic (non base paired G's) areas, unpaired ends, and telomeres (ends of chromosomes). In double stranded DNA, the modification is specific to these conformational areas. The oligonucleotides may be naturally occurring or synthetic. The

single-stranded DNA may also be derived from separation of duplex DNA. All forms of RNA are targets for reaction also.

A non-diffusible species is important for site-specificity in the target nucleotide. In contrast, certain complexes, e.g. Fe(II)-EDTA plus ligand, generate hydroxyl radicals which react with DNA non-specifically. In this type of non-preferred complex, the complex attaches to a binding site and generates a reactive species which diffuses with a loss of specificity. In the system described herein, the metal complex binds to a particular DNA site and the reactive agent is produced at that site when oxidant is present. A non-diffusible species is one which reacts with DNA before it is released from the specific binding site.

The complexes are used to modify or optionally nick DNA or nucleotides when used with an oxidant. Some examples of oxidizing agents include peracids such as peroxymonosulfate salts, e.g. potassium peroxymonosulfate (KHSO_5) which is commercially available under the trademark OXONE, magnesium monoperoxyphthalate (MMPP), and oxygen in combination with sulfite. Particularly preferred are MMPP or O_2 and sulfite. In physiological systems, a combination of oxygen and sulfite is preferred.

After oxidation, excess oxidant may be quenched with a high concentration of reductant such as sodium sulfite, and base treatment may be used for cleavage. Other quenching agents may include 10 mM EDTA or β -mercaptoethanol. Optionally, if scission of nucleic acid is desired, suitable bases are piperidine, N-butylamine and sodium hydroxide with a pH of about 9-13.

Reaction conditions include a temperature from about zero to about 100°C , from about 20°C to about 40°C preferred and 25°C - 37°C most preferred. Reaction time is at least about 30 seconds, from about 10 minutes to an hour preferred and from about 15 minutes to about 30 minutes most preferred. The reaction may be left for an extended period of time, e.g. up to 48 hours without adverse effects. The pH may be about 3-10, with 6.5-7.5 preferred.

For a reaction involving, for example, a nucleotide concentration of about 0.1 to about 50 picomoles of labelled 5'-ends, the metal complex may be used in an amount of from about 0.1 μM to about 10 mM, with from about 0.5 μM to about 100 μM preferred

and from about 1 μM to about 10 μM most preferred. The oxidant may be used in an amount of from about 0.1 μM to about 10 mM with about 50 μM to about 100 μM preferred. The ratio of metal complex to oxidant may range from about 99:1 to about 1:99. The oxidant is preferably in excess of the metal complex with a ratio of metal complex:oxidant ranging up to about 1:10,000, with from about 1:1 to about 1:50 preferred and 1:1 to 1:2 most preferred.

In vitro, the nucleotide or DNA is labelled in any suitable manner such as with radiolabel, and standard electrophoresis and autoradiography may be used to determine the level of modification.

The process of the invention for modifying DNA or sequencing is suitable for being attained using a kit. The kit may comprise a salicylimine containing metal complex to be used with an oxidant and optionally a base. The kit can supply a salicylimine containing metal complex in an amount of from about 0.01 to about 500 μM metal complex, preferably from about 0.1 to about 50 μM metal complex and most preferably about 10 to about 50 μM metal complex. An oxidant is selected from the group consisting of peracid, sulfite and O_2 . The preferred oxidants are oxone, MMPP, or a combination of sulfite and oxygen. The most preferred oxidant is MMPP. If the kit is to be used in an *in vivo* system, e.g., in tissue culture or in an animal, the oxidant is preferably provided by dissolved oxygen already present in the system, with the addition of sulfite. A base may also be provided, optionally and may be piperidine, N-butylamine or sodium hydroxide having a pH of about 9-13, if scission of the DNA is desired.

As used herein, "label" or "detectable label" refers to any molecule that is linked to the Ni-salen complex to provide a detectable signal. The label may be radioactive, chemiluminescent, a protein ligand, or an enzyme, or if a fluorescent group is used, a different fluorescent group may be used for different types of salicylimine containing nickel complexes. These fluorescent tags would have the property of having spectroscopically distinguishable emission spectra. If a protein ligand based detection method is desired, biotin or digoxigenin are examples of compounds that may be used as a detectable label. In the case of digoxigenin, enzymes linked to anti-digoxigenin antibodies may be used to detect the presence of digoxigenin. Enzyme-based detection assays include

for example, the use of peroxides and alkaline phosphatase, to obtain chromogenic substances. ELISA assays can be typically used in the practice of the instant invention.

However, it should be appreciated that the choice of a label depends merely on the preference of the person of ordinary skill in the art as the present invention can be performed with any detectable label at all.

The label can be linked anywhere on the salicylimine containing nickel complex. The label is preferably linked on the carbon backbone. More preferably, the label is linked adjacent to the salicylimine group. It should be understood that binding of the label to the complex can occur practically anywhere on the carbon backbone or a substituent of the backbone, directly or through a linker, such that a person of skill in the art would be able to attach the label to the nickel complex. Any group having a primary amine group could be a site of binding of the label. The person of skill in the art would know to attach a label to the nickel complex so that the label does not interfere with the binding of the salen group to the nucleic acid. For example, the attachment of the label should not destroy or hinder the formation of a square planar, 4-coordinate system.

As used herein, "linker" means any organic residue that serves to link the label to the nickel complex. Such a linker may be, without limitation, an alkyl group, or a compound having multiple CH_2 groups, an aryl group, an arylalkyl group, ethers, and so on. Other variations and substituents are possible, so long as the linker serves to connect the label to the nickel complex without hindering the binding of the complex to the nucleic acid.

Another object of the invention is to provide a method of purifying the nickel-complex-nucleotide adduct (covalently bound). If a biotin label is linked to the nickel complex, for example, then an avidin column may be used to bind the biotin label, and thereby purify the entire adduct, including the nucleic acid region. If another protein ligand is used as the label, then using a separation process comprising its corresponding antibody may be similarly used in an affinity chromatography procedure, for example. Other methods can be used so long as the method takes advantage of the covalent binding between the nickel-complex with a specific nucleotide, and that the nickel complex is directly or indirectly attached to a detectable label.

In another aspect of the invention, a library of metals, salicylaldehydes, oxidants and peptides may be screened to determine the best candidates for oxidative cross-linking to DNA. The salicylaldehyde-guanine adducts that have already been observed may be the subject of further mechanistic studies, with a view toward providing information relevant to tyrosine-DNA cross-linked lesions.

Another feature of the invention is that the Salen-LysHis metal-binding motif may be appended to the N terminus of peptide nucleic acids (PNA) for targeting specific sites in DNA or RNA oligonucleotide targets. Base specificity is examined, and the structures of potential C, T, and A adducts are determined using ESI-MS/MS from both oligomer and nucleoside model studies. Salicylaldehyde adducts may be further functionalized with biotin and fluorescein reagents or any other label.

With the Ni(Salen-LysHis) complex in hand as a redox active complex for oxidative coupling to DNA bases, we next sought the ideal recognition agent, or delivery vehicle, to target specific sites in nucleic acids. Peptide nucleic acid (PNA) oligomers have outstanding properties for nucleic acid binding and ease of synthesis. PNA now enjoys widespread use in biological chemistry since its initial discovery in 1991. Its applications range from genome cutting to drug delivery to *in situ* hybridization. Some of the pertinent features of PNA, include:

- PNAs obey Watson-Crick rules for base pairing and form a regular "antiparallel" duplex helical structure with DNA in that the N terminus of PNA normally binds with the 3' end of a DNA oligo. Parallel duplexes are also possible, but less stable. Crystal structures show conformations similar to B-form DNA for PNA-DNA. PNA-RNA has been analyzed by NMR spectroscopy and indicates an A-like helix for the RNA.
- PNAs have high binding affinity for DNA because of their neutral charge. A PNA-DNA duplex shows a melting temperature typically 1° C higher per base pair than a DNA-DNA duplex. The stability of PNA-RNA duplexes is even higher—about 1.5° C per base pair higher than RNA-RNA duplexes.
- A consequence of the higher T_m 's is that shorter sequences may be used to impart high stability to a duplex. Furthermore, a PNA oligomer will displace a DNA

strand from a duplex in order to bind to its target DNA.

- PNA is less forgiving of base pair mismatches than is DNA. A single mismatch typically leads to an 8-20° C lowering of the T_m in a PNA-DNA duplex. This means that high sequence selectivity is ensured.
- PNA forms triple helical structures but these are primarily restricted to polypyrimidine sequences. $(PNA)_2$ -DNA is more stable than $PNA-(DNA)_2$.
- PNA is readily synthesized using either fmoc or Boc methodology on solid support; both types of the 4 protected base monomers are commercially available. The methodology is completely compatible with peptide synthesis and several peptide-PNA conjugates are known.
- PNA shows high chemical stability to HF, ammonia and piperidine and remarkably high biostability in both human serum and cell extracts.
- The cellular uptake of PNAs is low although DNA/PNA chimeras are taken up by Vero cells and NIH3T3 cells at low (1 μ M) extracellular concentration.
- Applications of PNA currently include diagnostic methods for screening for genetic mutations, probes in pre-gel hybridization techniques (an alternative to Southern blotting) and agents for assisting MALDI-TOF analysis of single-nucleotide polymorphisms in the human genome.

The following examples are offered by way of illustration of the present invention, and not by way of limitation.

EXAMPLES

Example 1 - Synthesis and Activity of Ni(II)-salen-biotin complex 2

The oxidation and coordination chemistry of transition metal ions offer substantial opportunities for selective recognition and modification of nucleic acids. (Sigel, H., Sigel, A. Eds.; Marcel Dekker: *Metal Ions in Biological Systems*, Vols. 32 and 33; Dekker: New York, 1996.) (Pyle, A. M; Barton, J. K. *Prog. Inorg. Chem.* 1990, 38, 413-475.) Certain nickel complexes in particular have demonstrated alternative abilities to oxidize and couple with highly accessible guanine residues in the presence of O_2 and sulfite (Muller, J. G.;

Hickerson, R. P.; Perez, R. J.; Burrows, C. J. *J. Am. Chem. Soc.* 1997, *119*, 1501-1506.) (Stemmler, A. J.; Burrows, C. J. *J. Am. Chem. Soc.* 1999, *121*, 6956-6957.) or peracids such as monoperoxysulfate or monoperoxyphthalate. (Burrows, C. J.; Rokita, S. E. *Acc. Chem. Res.* 1994, *27*, 295-301.) (Burrows, C. J.; Rokita, S. E. In *Metal Ions in Biological Systems*, Vol. 32; Sigel, H., Sigal, A. Eds. Dekker: New York, 1996; Chapter 18, pp. 537-560.) (Rokita, S. E.; Burrows, C. J. in *Current Protocols in Nucleic Acid Chemistry*; Beaucage, S. I., Bergstrom, D. E., Glick, G., Jones, R. A. Eds. Wiley, New York, 2000; Chapter 6.4.) (Muller, J. G.; Paikoff, S. J.; Rokita, S. E.; Burrows, C. J. *J. Inorg. Biochem.* 1994, *54*, 199-206.) (Muller, J. G.; Kayser, L. A.; Paikoff, S. J.; Duarte, V.; Tang, N.; Perez, R. J.; Rokita, S. E.; Burrows, C. J. *Coord. Chem. Rev.* 1999, *185-186*, 761-774.) Adducts formed between guanine and nickel complexes based on the salen ligand (ethylene-N-N'-bis(salicyaldimine)) strongly inhibit polynucleotide elongation catalyzed by reverse transcriptase and DNA polymerase and consequently allow for sensitive detection through primer extension assays. (Muller, J. G.; Paikoff, S. J.; Rokita, S. E.; Burrows, C. J. *J. Inorg. Biochem.* 1994, *54*, 199-206.) (Muller, J. G.; Kayser, L. A.; Paikoff, S. J.; Duarte, V.; Tang, N.; Perez, R. J.; Rokita, S. E.; Burrows, C. J. *Coord. Chem. Rev.* 1999, *185-186*, 761-774.) (Woodson, S. A.; Muller, J. G.; Burrows, C. J.; Rokita, S. E. *Nucleic Acids Res.* 1993, *21*, 5524-5526. Pan, J.; Woodson, S. A. *J. Mol. Biol.* 1998, *280*, 597-609.)

The salen ligand serves two functions by activating the redox chemistry of the bound Ni(II) and generating a ligand-centered radical for addition to guanine. (Muller, J. G.; Paikoff, S. J.; Rokita, S. E.; Burrows, C. J. *J. Inorg. Biochem.* 1994, *54*, 199-206.) (Muller, J. G.; Kayser, L. A.; Paikoff, S. J.; Duarte, V.; Tang, N.; Perez, R. J.; Rokita, S. E.; Burrows, C. J. *Coord. Chem. Rev.* 1999, *185-186*, 761-774.) (Goldsby, K. A., *J. Coord. Chem.* 1988, *19*, 83-90.) (Goldsby, K. A., Blaho, J. K.; Hoferkamp, L. A. *Polyhedron* 1989, *8*, 113-115.) Direct coordination between a transient Ni(III) intermediate and accessible N7 sites on guanine appear to enhance the specificity. (Shih, H.-C.; Kassahun, H.; Burrows, C. J.; Rokita, S. E. *Biochemistry* 1999, *38*, 15034-15042) Previous investigations have focused on the water-soluble salen complex 1, NiTMAPES (Fig. 1), but a wide range of useful derivatives can be envisioned including a salen-peptide

hybrid. (Stemmler, A. J.; Burrows, C. J. *J. Am. Chem. Soc.* 1999, 121, 6956-6957.) (Muller, J. G.; Paikoff, S. J.; Rokita, S. E.; Burrows, C. J. *J. Inorg. Biochem.* 1994, 54, 199-206.) (Muller, J. G.; Kayser, L. A.; Paikoff, S. J.; Duarte, V.; Tang, N.; Perez, R. J.; Rokita, S. E.; Burrows, C. J. *Coord. Chem. Rev.* 1999, 185-186, 761-774) (Shearer, J. M.; Rokita, S. E. *Bioorg. Med. Chem. Lett.* 1999, 9, 510-504.) These nickel salens appear unique in their ability to couple with their targets rather than promote direct strand scission as common to the salen complexes of Mn, Co and Cu. (Muller, J. G.; Kayser, L. A.; Paikoff, S. J.; Duarte, V.; Tang, N.; Perez, R. J.; Rokita, S. E.; Burrows, C. J. *Coord. Chem. Rev.* 1999, 185-186, 761-774.)

Although footprinting well-defined polynucleotides with NiTMAPES may be routine with piperidine cleavage or primer extension, obtaining the actual products of coupling can be an arduous task. As the heterogeneity of the system increases, even footprinting becomes difficult. This type of problem is common in molecular biology, and its solution often relies on a broad range of techniques based on biotin. Labeling, detecting and purifying any biotinylated species regardless of its complexity is made possible by the extraordinary affinity of biotin for avidin and streptavidin. (Launer, H. F.; Fraenkel-Conrat, H. *J. Biol. Chem.* 1951, 193, 125-132.) (Green, N. M. *Biochem. J.* 1963, 89, 585-620. Green, N. M. *Adv. Protein Chem.* 1975, 29, 85-133.) (Diamonds, E. P.; Christopoulos, T. K. *Clin. Chem.* 1991, 37, 625-636.) (Wilchek, M.; Edward, E. A., Eds. *Methods in Enzymol.* 1990, 184, entire volume.) The properties of a combined Ni(salen)-biotin conjugate should then have the potential to diagnosis and isolate genomic sequences containing noncanonical structures of guanine. The first synthesis of such a conjugate and its initial characterization with a model oligonucleotide is described herein.

Early synthetic targets were designed in direct analogy to TMAPES and were expected to support a range of strategies for biotinylation. However, the instability and low reactivity of the essential intermediates precluded this scheme. These problems were avoided in a subsequent approach that relied on a triamine first developed by the laboratory of Bailly for use in construction of Cu(salen) derivatives. (Routier, S.; Bernier, J.-L.; Waring, M. J.; Colson, P.; Houssier, C.; Bailly, C. *J. Org. Chem.* 1996, 61, 2326-2331.) (Routier, S.; Cotellet, N.; Catteau, J.-P.; Bernier, J. -L.; Waring, M. J.; Riou, J.-F.; Bailly,

C. Bioorg. Med. Chem. 1996, 4, 1185-1196.) N α -Z-N ϵ -BOC-L-lysine **3** was converted to its nitrile derivative **4** as described previously (Figure 2). (Routier, S.; Bernier, J.-L.; Waring, M. J.; Colson, P.; Houssier, C.; Bailly, C. *J. Org. Chem.* 1996, 61, 2326-2331.) (Routier, S.; Cotellet, N.; Catteau, J.-P.; Bernier, J. -L.; Waring, M. J.; Riou, J.-F.; Bailly, C. *Bioorg. Med. Chem.* 1996, 4, 1185-1196.) A subsequent procedure requiring hydrogenation under high pressure was avoided by use of two sequential hydrogenations in the presence of Raney nickel and then Pd/C to yield the diamine **5** (see Example 2 for experimental details). The BOC-protected salen derivative **6** was formed by condensation of this diamine under N₂ with two equivalents of 2,4-dihydroxybenzaldehyde in the presence of Ni(OAc)₂. Deprotection by standard conditions (dry TFA/CH₂Cl₂) and coupling with the commercially available N-hydroxysuccinimide ester of biotin (BNHS) produced the neutral and water-insoluble derivative **7**. The obligatory solubility in water was achieved through alkylation of the two free phenolic oxygens with 3-bromopropyltrimethyl ammonium bromide. (Kayser, L. A., M. S. thesis, University of Utah, 1997.) The desired perchlorate salt of **2** was precipitated by addition of KClO₄ and recrystallized from MeOH/Et₂O. Data from electrospray mass spectroscopy as well as ¹H- and ¹³C-NMR were consistent with the desired product.

Incubation of a model oligonucleotide (18' mer, one central G) with the Ni(salen)-biotin conjugate **2** (50 μ M) or the magnesium salt of monoperoxyphthalic acid (MMPP) (120 μ M) in 10 mM sodium phosphate pH 7 generated no apparent products as detected by denaturing polyacrylamide gel electrophoresis (Figure 3, lanes 2 and 3). However, their combined presence produced a high molecular weight DNA adduct in approximately 60% yield (Figure 3, lane 4). This yield decreased to 5% when the ionic strength of the mixture was increased by addition of 100 mM NaCl. In either case, reaction remained specific for the sole guanine residue as indicated below. The biotinylated DNA was conveniently separated from its parent oligonucleotide with a monomeric avidin matrix (Pierce) and desalted with reverse-phase (C-18) chromatography (Figure 3, lane 5). Subsequent treatment with hot piperidine was investigated since this condition had previously induced a diagnostic strand scission of adducts formed by NiTMAPES **1**. (Muller, J. G.; Paikoff, S. J.; Rokita, S. E.; Burrows, C. J. *J. Inorg. Biochem.* 1994, 54, 199-206.) (Muller, J. G.;

Kayser, L. A.; Paikoff, S. J.; Duarte, V.; Tang, N.; Perez, R. J.; Rokita, S. E.; Burrows, C. J. *Coord. Chem. Rev.* 1999, 185-186, 761-774.) The adduct of conjugate 2 demonstrated equivalent lability in the presence of hot piperidine. The resulting oligonucleotide fragment co-migrated with the guanine standard produced by Maxam-Gilbert sequencing (Figure 3, lanes 6 and 7, respectively). (Maxam, A. M.; Gilbert, W. *Methods Enzymol.* 1980, 65, 499-560.) As expected, the parent oligonucleotide exhibited no sensitivity to piperidine (Figure 3, lane 8).

Composition of the isolated adduct was confirmed by mass spectral analysis. Electrospray mass spectroscopy identified a major species of mass 6377 that is consistent with the oligonucleotide + Ni(salen) + oxygen. The additional oxygen was anticipated from MMPP-dependent oxidation of the biotin sulfide. Incubation of the unconjugated biotin under conditions analogous to the DNA experiments above led to formation of biotin sulfoxide as indicated by comparison to a standard produced by known reaction with H₂O₂. (Melville, D. B. *J. Biol. Chem.* 1954, 208, 495-500.) This oxidation did not significantly interfere with avidin affinity chromatography since the major determinant for recognition is the ureido group of biotin. (Weber, P. C.; Ohlendorf, D. H.; Wendoloski, J. J.; Salemme, F. R. *Science* 1989, 243, 85-88.)

The ability of the nickel complex to deliver a biotin tag to accessible guanine residues allows for the isolation and characterization of noncanonical structures in complex systems such as chromatin. Few alternatives are currently available that share both a dependable conformational specificity and an easy assimilation into the wide ranging and powerful techniques based on biotin recognition.

Example 2 - Materials and methods used in the synthesis of Ni(Salen)-Biotin Conjugates 2 depicted in Fig.2

General. ¹H and ¹³C NMR spectra were recorded on Bruker DPX 400 and 500 spectrometers. Chemical shifts are reported as δ values relative to internal standards such as CHCl₃, CH₃OH or CH₃CN. Mass spectra were determined using VG 7070E (FAB) and Micromass Quattro II triple quadrupole (electrospray) instruments. Column chromatography was performed with Merck kieselgel 60 silica (230 ASTM). CH₂Cl₂ was

distilled from calcium hydride; EtOH was distilled from Mg; DMF was distilled from MgSO_4 ; and TFA was distilled from P_2O_5 . Other solvents and chemicals were used as received.

(S)-N'-(Benzyloxycarbonyl)-1,5-diaminopentane. (S)-1-Cyano-N1-(benzyloxycarbonyl)-N5-(*tert*-butyloxycarbonyl)-1,5-diaminopentane (Routier, S.; Bernier, J.-L.; Waring, M. J.; Colson, P.; Houssier, C.; Bailly, C. *J. Org. Chem.* **1996**, *61*, 2326-2331. (b) Routier, S.; Cotellet, N.; Catteau, J.-P.; Bernier, J. -L.; Waring, M. J.; Riou, J.-F.; Bailly, C. *Bioorg. Med. Chem.* **1996**, *4*, 1185-1196) **4** (500 mg, 1.38 mmol) and Raney-Ni (1 g) were added to ammonia saturated MeOH (20 mL) and stirred under hydrogen gas (6.8 atm) at 50°C. After 72 hrs, this mixture was filtered. Solvent was removed under reduced pressure to yield the desired product (colorless oil, 360 mg, 72%). ^1H NMR (CDCl_3) δ 1.4-1.6 (m, 16H), 1.78 (m, 2H), 2.70-3.10 (m, 4H), 4.8 (bs, 2H), 5.1 (bs, 2H), 7.31 (m, 5H). HRMS (FAB) Calcd ($\text{M}+\text{H}^+$) $\text{C}_{19}\text{H}_{32}\text{N}_3\text{O}_4$: 366.2393, found: 366.2401.

(S)-6-(*tert*-Butyloxycarbonyl)-1,2,6-triaminohexane (5). A solution of the triamine above (350 mg, 0.96 mmol) in MeOH (100 mL) was combined with Pd/C (10%, 40 mg) and stirred under hydrogen gas (1 atm) over night at room temperature. The mixture was filtered and evaporated under reduced pressure to yield a crude pink solid (180 mg, 82%) that was used below without purification. ^1H NMR (CDCl_3) δ 1.40-1.60 (m, 15H), 1.88 (m, 2H), 2.70-3.10 (m, 4H), 4.80 (m, 4H). FAB m/z 232 ($\text{M}+\text{H}^+$).

(S)-N, N'-Bis(3-hydroxysalicylidene)-6-(*tert*-butyloxycarbonyl)-1,2,6-triaminohexane nickel complex (6). (Routier, S.; Bernier, J.-L.; Waring, M. J.; Colson, P.; Houssier, C.; Bailly, C. *J. Org. Chem.* **1996**, *61*, 2326-2331. (b) Routier, S.; Cotellet, N.; Catteau, J.-P.; Bernier, J. -L.; Waring, M. J.; Riou, J.-F.; Bailly, C. *Bioorg. Med. Chem.* **1996**, *4*, 1185-1196) 2,4-Dihydroxybenzaldehyde (239 mg, 1.73 mmol) and **5** (200 mg, 0.866 mmol) were dissolved in dry EtOH (45 mL) and heated to reflux under N_2 for 30 min. Nickel acetate (861 mg, 3.46 mmol) was then added and heating was continued for 3 hours. During this period, the clear solution became orange. The solvent was evaporated under reduced pressure, and the remaining solid was subjected to silica chromatography using a solvent

system of $\text{CH}_2\text{Cl}_2/\text{MeOH}$ (10/1). The desired product 6 was isolated in 78% yield (357 mg). ^1H NMR (CD_3OD) δ 1.26-1.52 (m, 13H), 1.80 (m, 2H), 2.97-3.04 (m, 3H), 3.15-3.16 (m, 1H), 3.63 (m, 1H), 6.09 (m, 2H), 6.23 (s, 2H), 7.00 (d, $J_1 = 8.4$ Hz, 1H), 7.05 (d, $J_2 = 8.8$ Hz, 1H), 7.44 (s, 1H), 7.47 (s, 1H). ^{13}C NMR (CD_3OD) δ 24.2, 28.8, 30.8, 36.7, 41.1, 63.6, 69.4, 79.9, 105.4, 107.4, 107.5, 115.8, 116.1, 155.4, 135.7, 158.6, 162.1, 163.1, 164.5, 164.6, 166.7, 166.9. HRMS(FAB) Calcd ($\text{M}+\text{H}^+$) $\text{C}_{25}\text{H}_{32}\text{N}_3\text{O}_6\text{Ni}$: 528.1644, found: 528.1628.

(S)-N, N'-Bis(3-hydroxysalicylidene)-1,2,6-triaminohexane, TFA, nickel complex. (Routier, S.; Bernier, J.-L.; Waring, M. J.; Colson, P.; Houssier, C.; Bailly, C. *J. Org. Chem.* 1996, 61, 2326-2331. Routier, S.; Cotellet, N.; Catteau, J.-P.; Bernier, J. -L.; Waring, M. J.; Riou, J.-F.; Bailly, C. *Bioorg. Med. Chem.* 1996, 4, 1185-1196.) TFA (75 μL) was added dropwise over 30 min to a solution of 6 (10 mg, 0.0189 mmol) in CH_2Cl_2 (2.0 mL) at room temperature under N_2 . After stirring for an additional 30 min, the solvent was removed under reduced pressure. EtOH (5 mL) was twice added and evaporated to help remove residual TFA. The crude product was dissolved in MeOH and precipitated by addition of CH_2Cl_2 to yield an orange solid (6.9 mg, 86%). ^1H NMR (CD_3OD) δ 1.39 (m, 8H), 2.88 (m, 2H), 3.59 (m, 1H), 6.09 (m, 1H), 6.22 (s, 1H), 6.40 (d, $J=8.2$ Hz, 1H), 7.02 (m, 1H), 7.06 (m, 1H), 7.45 (m, 2H). ^{13}C NMR (CD_3OD) δ 22.5, 26.7, 27.1, 35.1, 39.0, 62.2, 67.9, 103.9, 106.1, 106.2, 108.4, 114.3, 114.6, 134.0, 160.9, 161.8, 163.1, 165.5. HRMS (FAB) Calcd ($\text{M}+\text{H}^+-\text{TFA}$) $\text{C}_{20}\text{H}_{24}\text{N}_3\text{O}_4\text{Ni}$: 428.1120, found: 428.1161.

(S)-N, N'-Bis(3-hydroxysalicylidene)-6-biotinyl-1,2,6-triaminohexane nickel complex (7). A mixture of the Boc-protected TFA salt above (20 mg, 0.047 mmol), N-hydroxysuccinimide biotin ester (16 mg, 0.047 mmol) and triethylamine (8 μL) in dry DMF (4 mL) was stirred at 4° C for 72 hours. Solvent was removed under reduced pressure, and the solid residue was subjected to silica gel chromatography using a solvent system of $\text{CH}_2\text{Cl}_2/\text{MeOH}$ (5/1). The desired product 7 was isolated as an orange solid (6.4 mg, 22%). ^1H NMR (CD_3OD) δ 1.26-1.57 (m, 12H), 2.11 (m, 2H), 2.66 (m, 1H), 2.88 (m, 1H), 3.01 (m, 1H), 3.18 (m, 3H), 3.60 (m, 2H), 4.30 (m, 1H), 4.48 (m, 1H), 6.11 (m, 2H), 6.24 (s, 2H), 7.07 (m, 2H), 7.49 (s, 1H), 7.51 (s, 1H). ^{13}C NMR (CD_3OD) δ 24.2, 26.9,

29.4, 29.7, 30.2, 30.8, 36.6, 36.8, 40.0, 41.1, 57.1, 61.6, 63.4, 63.7, 69.3, 105.4, 107.5, 107.6, 115.8, 116.1, 135.4, 135.7, 162.2, 163.2, 164.6, 166.7, 167.0, 176.1 HRMS (FAB) Calcd (M+H⁺) C₃₀H₃₈N₅O₆Ni: 654.1896, found: 654.1866.

(S)-N, N'-Bis(3-(3-trimethylaminopropoxy)salicylidene)-6-biotinyl-1,2,6-

triaminohexane nickel complex (2). 3-Bromopropyltrimethylammonium bromide (15.9 mg, 0.061 mmol) and K₂CO₃ (8.6 mg) were added to a solution of **7** (10 mg, 0.0153 mmol) in DMF (3 mL) and stirred at room temperature for three days (Kayser, L. A., M. S. thesis, University of Utah, 1997). The solvent was removed under reduced pressure to yield a crude solid. The solid was dissolved in water (1 mL) and a solution of KClO₄ (1 M) was slowly added dropwise until the first indication of precipitation was evident. This mixture was cooled to 4° C overnight. The resulting precipitate was collected by filtration, redissolved in MeOH and precipitated with Et₂O. This procedure was repeated twice. The precipitate was isolated by filtration and recrystallized twice with MeOH/Ether. The desired product **2** was obtained as an orange solid (12.8 mg, 80%). ¹H NMR (CD₃CN) δ 1.25-1.50 (m, 18H), 1.75 (m, 1H), 2.03 (m, 1H), 2.70 (m, 1H), 2.80 (m, 1H), 3.03 (bs, 18H), 3.11 (m, 4H), 3.40 (m, 2H), 3.56 (m, 1H), 4.04 (m, 2H), 4.23 (m, 1H), 4.38 (m, 1H), 4.95 (s, 1H), 5.35 (bs, 1 H), 6.16-6.29 (m, 4H), 7.12 (m, 2H), 7.49 (s, 2H). ¹³C NMR (CD₃CN) δ 23.77, 26.54, 28.89, 28.95, 29.76, 30.35, 36.05, 36.41, 39.26, 41.20, 54.01, 56.36, 60.70, 62.42, 63.40, 64.96, 65.16, 69.00, 103.14, 103.27, 106.44, 134.86, 135.18, 161.78, 162.78, 163.74, 164.23, 173.75. ESI-MS: *m/z* 954.4 ([M⁺-ClO₄], 3%), 427.7 ([M⁺-2(ClO₄)]/2, 100%).

Biotin-S-oxide. MMPP (49 mg, 0.10 mmol) was added to a solution of biotin (10 mg, 0.041 mmol) in 10 mM phosphate pH 7.0 (1 mL) and incubated at room temperature for 30 min. After removing the solvent under reduced pressure, crude product was dissolved in a solution of glacial acetic acid (20 μL) and absolute ethanol (1 mL) and maintained at 4° C for 2 days. The crystalline product was filtered, washed and dried to yield 9.2 mg (85 %). m.p. 239-242° C ((Melville, D. B. *J. Biol. Chem.* **1954**, *208*, 495-500) 238-241° C).

DNA experiments. The oligonucleotide 5'-d(AAAATATCAGATCTAAAA) was prepared by automated solid-phase synthesis, purified using a NAPTM-25 column (Pharmacia Biotech) and 5' end-labeled with ³²P using T4 kinase and gamma-[³²P]-ATP. DNA (11 μM, 6 nCi) was mixed with the Ni(salen)-biotin conjugate **2** (50 μM) in sodium phosphate (10 mM, pH 7.0) and incubated under ambient conditions for 10 min. Reaction was then initiated by addition of MMPP (120 μM) to a final volume of 50 μL. After further incubation for 30 min, a loading buffer (8 M urea) was added, and the samples were directly analyzed by denaturing polyacrylamide gel electrophoresis (20%) and phosphoimager.

The DNA adduct formed by the Ni(salen)-biotin conjugate was isolated on a preparative scale using non-radiolabeled DNA. Reaction conditions were identical to those described above. Only the final reaction volume was increased to 2.5 mL. After incubation, this volume was reduced by 20% under vacuum and then applied directly to a monomeric avidin column (2 mL, Pierce). Unmodified DNA was eluted with a solution of 150 mM NaCl in 100 mM sodium phosphate pH 7.2. The DNA conjugate was eluted with 2 mM biotin in the solution above as described by the manufacturer. The salts and free biotin of the elution buffer were removed by reverse phase (C-18, Sep-Pak) chromatography using water and then 30% CH₃CN in water as solvents. The isolated conjugate was lyophilized, dissolved in ammonium acetate (10 M, 50 μL) and precipitated with ethanol/isopropanol prior to alternative radiolabeling and analysis by electrospray mass spectrometry. Strand fragmentation of the adduct was accomplished by resuspending a dried and radiolabeled sample in piperidine (0.2 M, 60 μL), heating to 90°C for 30 min and finally lyophilizing. Residual piperidine was removed by repeated cycles of resuspending (water) and drying the solid pellet.

Example 3 - Preparation of Salen-XaaHis Motif for Metal-Mediated Oxidative DNA-Peptide Cross-Linking

Metallopeptide motifs of the N-terminal sequence XaaXaaHis, wherein histidine resides in the third position of the peptide, were first described for albumins (Harford, C.; Sarkar, B. *Acc. Chem. Res.* 1997, 30, 123-130.) and later utilized as redox-active

bioconjugates for DNA cleavage (Mack, D. P.; Iverson, B. L.; Dervan, P. B. *J. Am. Chem. Soc.* 1988, *110*, 7572-7574.) (Shullenberger, D. F.; Eason, P. D.; Long, E. C. *J. Am. Chem. Soc.* 1993, *115*, 11038-11039.) (Nagaoka, M.; Hagihara, M.; Kuwahara, J.; Sugiura, Y. *J. Am. Chem. Soc.* 1994, *116*, 4085-4086.) and protein-protein cross-linking. (Brown, K. C.; Yang, S.-H.; Kodadek, T. *Biochemistry* 1995, *34*, 4733-4739.) Metallosalens are another class of tetradentate ligands that have seen applications to DNA chemistry. (Burrows, C. J.; Rokita, S. E. *Acc. Chem. Res.* 1994, *27*, 295-301.) (Morrow, J. T.; Kolasa, K. A. *Inorg. Chim. Acta* 1992, *195*, 245.) (Gravert, D. J.; Griffin, J. H. *Inorg. Chem.* 1996, *35*, 4837-4847.) (Routier, S.; Bernier, J.-L.; Catteau, J.-P.; Bailly, C. *Bioorg. Med. Chem. Lett.* 1997, *7*, 63-66.) (Routier, S.; Bernier, J.-L.; Waring, M. J.; Colson, P.; Houssier, C.; Bailly, C. *J. Org. Chem.* 1996, *61*, 2326-2331.) The juxtaposition of these two motifs leads to a Schiff-base metallopeptide hybrid that might combine the molecular recognition features of a peptide with the chemical reactivity of salicylaldimine complexes. Toward this end, we describe the synthesis and characterization of a prototypical member of this new "salen-XaaHis" ligand class and an example of DNA-peptide cross-linking.

Bidentate and tridentate Schiff-base adducts to peptides have been previously described, including complexes of a broad range of transition metals. (Sakurai, T.; Hongo, J.-I.; Nakahara, A. *Inorg. Chim. Acta* 1980, *46*, 205-210.) (Cole, B. M.; Shimizu, K. D.; Krueger, C. A.; Harrity, J. P. A.; Snapper, M. L.; Hoveyda, A. H. *Angew. Chem. Int. Ed. Engl.* 1996, *35*, 1668-1671.) These chelates have shown applications as diverse as asymmetric addition of CN^- to aldehydes, (Mori, A.; Nitta, H.; Kudo, M.; Inoue, S. *Tetrahedron Lett.* 1991, *32*, 4333-4336.) epoxides (Shimizu, K. D.; Cole, B. M.; Krueger, C. A.; Kuntz, K. W.; Snapper, M. L.; Hoveyda, A. H. *Angew. Chem. Int. Ed. Engl.* 1997, *36*, 1704-1707.) and imines. (Sigman, M. S.; Jacobsen, E. N. *J. Am. Chem. Soc.* 1988, *110*, 4901-4902.) The majority of these complexes involve tridentate chelation via the phenolate oxygen, the imine nitrogen and the first amide bond of the peptide. By including a histidine residue in position 2 of the peptide, we now extend the coordination environment to a square-planar, tetradentate mode while still allowing for C-terminal elaboration of the peptide. See Fig. 4, complexes 8 and 9.

Arginine (Arg) was chosen for position "Xaa" of the salen-XaaHis motif in order to

impart water solubility and some degree of DNA affinity to the complex. [Ni(salen-ArgHis)]OAc, **8** (Fig. 4), was synthesized by addition of *bis*-(salicylaldehyde)Ni to a pH 8, 1:2 ethanol/water solution of the dipeptide, Arg-His bearing a C-terminal carboxamide. Orange microcrystals were obtained from this solution after 4 h at room temperature; crystals suitable for x-ray crystallographic analysis were obtained by recrystallization from 2:1 methanol/water. The crystal structure of **8**, shown in Figure 5, depicts the tetradentate nature of the salen-ArgHis ligand as predicted. (Crystal data (293 K) for [Ni(salen-ArgHis)]OAc·3H₂O, **8**: NiC₂₁H₃₄N₈O₈, M_r=585.27, monoclinic, space group C2, $a=17.0767(3)$, $b=9.1342(3)$, $c=16.3666(5)$ Å, $\alpha=90$, $\beta=101.4810(16)$, $\gamma=90^\circ$, $V=2501.81(12)$ Å³, $Z=4$, GOF = 1.089. Final R values ($I>2\sigma I$): $R1=0.0334$, $wR2=0.0969$.) The coordination geometry about the nickel ion is square planar as evidenced by the very small deviation (0.001 Å) from the plane defined by the four ligand donor atoms. The overall charge on the molecule is positive due to the guanidino moiety of arginine; an acetate counter ion is present in a hydrogen-bonded network of 3 water molecules associated with the periphery of the ligand.

Consistent with the crystal structure, the solution structure of [Ni(salen-ArgHis)]⁺ appears to be that of a square-planar, diamagnetic species based on the NMR and uv-visible absorption spectra (d-d transition at 398 nm, $\epsilon=1,930$ cm⁻¹M⁻¹). Cyclic voltammetric studies of **8** (Fig. 4) as a 1 mM aqueous solution (pH 7, 10 mM NaP_i, 100 mM NaCl) showed a largely irreversible oxidation at $E_p \sim +0.85$ V vs. Ag/AgCl (scan rate = 100 mV s⁻¹), although some evidence for a small reduction at 0.80 V could be detected ($i_{pc}/i_{pa} \sim 0.4$). This behavior is consistent with ligand-centered oxidation due to the facile formation of a phenolate radical, as is typically observed in Ni(salen) complexes.

The ligand-centered redox chemistry of the new Ni(salen-XaaHis) complex immediately suggested that it might participate in an oxidative cross-link with DNA. We previously reported that the water-soluble salen complex, Ni(TMAPES), **9**, formed an adduct to guanine residues under oxidative conditions. (Burrows, C. J.; Rokita, S. E. *Acc. Chem. Res.* 1994, 27, 295-301.) (Muller, J. G.; Paikoff, S. J.; Rokita, S. E.; Burrows, C. J. *J. Inorg. Biochem.* 1994, 54, 199-206.) (Burrows, C. J.; Muller, J. G. *Chem. Rev.* 1998, 98, 1109-1151.) In those studies, treatment of DNA or RNA with **9** in the presence of KHSO₅,

led to the formation of a guanine adduct that could be characterized by electrospray ionization MS (ESI-MS), (Muller, J. G.; Kayser, L. A.; Paikoff, S. J.; Duarte, V.; Tang, N.; Rokita, S. E.; Burrows, C. J. *Coord. Chem. Rev.* 1999, 185-186, 761-774) primer extension assays, (Woodson, S. A.; Muller, J. G.; Burrows, C. J.; Rokita, S. E. *Nucleic Acids Res.* 1993, 21, 5524-5525.) and PAGE analysis of short oligonucleotides. (Muller, J. G.; Paikoff, S. J.; Rokita, S. E.; Burrows, C. J. *J. Inorg. Biochem.* 1994, 54, 199-206.) The participation of the phenolate moiety in **9** as part of the cross-link was confirmed through substituent effect studies, (Muller, J. G.; Kayser, L. A.; Paikoff, S. J.; Duarte, V.; Tang, N.; Rokita, S. E.; Burrows, C. J. *Coord. Chem. Rev.* 1999, 185-186, 761-774) and the modified guanine was found to be a piperidine-labile DNA cleavage site (Muller, J. G.; Paikoff, S. J.; Rokita, S. E.; Burrows, C. J. *J. Inorg. Biochem.* 1994, 54, 199-206.) (Muller, J. G.; Kayser, L. A.; Paikoff, S. J.; Duarte, V.; Tang, N.; Rokita, S. E.; Burrows, C. J. *Coord. Chem. Rev.* 1999, 185-186, 761-774.)

Studies reported herein show that the cross-linking behavior of a phenolate moiety can now be incorporated into the N-terminal motif of a peptide. As shown in Fig. 6, treatment of a 5'-[³²P]-end-labeled 12-mer oligodeoxynucleotide with **8** (100 μ M) and KHSO₃ (20 or 60 μ M, lane 3 or 4), followed by quenching with HEPES + EDTA, led to a high molecular weight band observable by PAGE. Interestingly, **8** appears to share with nickel peptides (Muller, J. G.; Hickerson, R. P.; Perez, R. J.; Burrows, C. J. *J. Am. Chem. Soc.* 1997, 119, 1501-1506.) and nickel salens the ability to catalyze HSO₃⁻ formation *in situ* via autoxidation of sulfite. Thus, cross-linking of **8** to DNA was even more effective when KHSO₃ was replaced with Na₂SO₃ (0.1 or 1 mM, lane 5 or 6). Sulfite can be used in greater concentration than HSO₃⁻, an oxidant that would lead to indiscriminant background oxidation of DNA if used in concentrations >100 μ M. Control studies confirmed that no high molecular weight band was observed if Ni²⁺ + ArgHis (lacking salicylaldehyde) was used in place of **8**. The position on the gel of the high molecular-weight bands from **8** are significantly lower than that from **9** (whose DNA cross-link is shown for comparison in Fig. 6, lane 9), suggesting that the charge and molecular weight of this adduct are different. It is noted that **8** was used in higher concentration than **9** because of its lower electrostatic attraction for DNA. Extension of the peptide to include a DNA-binding moiety should

greatly reduce the required concentration of metal complex.

Treatment of the [Ni(salen- ArgHis)]⁺-modified DNA with 0.2 M piperidine at 90° C for 30 min. led to substantial cleavage, but only at the single G residue of the oligomer (see Figure 7). Several other oligonucleotide sequences were used, and all gave the same G chemistry as described here. Control studies demonstrated that no other base modifications, such as imidazolone formation, (Cadet, J.; Berger, M.; Buchko, G. W.; Joshi, P. C.; Raoul, S.; Ravanat, J.-L. *J. Am. Chem. Soc.* 1994, 116, 7403-7404.) were produced by these reaction conditions. The piperidine sensitivity of the DNA adduct suggests that the cross-link has likely formed to either N7 or C8 of G, since both of these sites of modification typically lead to piperidine-induced strand scission. (Burrows, C. J.; Muller, J. G. *Chem. Rev.* 1998, 98, 1109-1151.) Indeed, arylation of guanine at both of these positions has been observed for the phenolic A-ring of estrogen. (Akanni, A.; Tabakovic, K.; Abul-Hajj, Y. J. *Chem. Res. Toxicol.* 1997, 10, 477-481.) Mass spectral analysis provided further information on the site and identity of adduct formation; isolation of the major 8-DNA adduct from the gel and precipitation with NH₄OAc led to a sample suitable for ESI-MS. The final spectrum showed a single product of mass 3739 consistent with DNA + salicylaldehyde - 10 Daltons (see Figure 8). The overall loss of 10 mass units corresponds to the now generally observed phenomenon of further oxidation of any C8-modified guanine adduct leading to a guanidinohydantoin product after hydration and decarboxylation of C6 of the purine. (Duarte, V.; Muller, J. G.; Burrows, C. J. *Nucleic Acids Res.* 1999, 27, 496-502.) (Poje, M.; Sokolic-Maravic, L. *Tetrahedron* 1988, 44, 6723-6728.) (Shibutani, S.; Gentles, R. G.; Iden, C. R.; Johnson, F. J. *Am. Chem. Soc.* 1990, 112, 5667-5668.) Thus, the DNA partner in cross-link formation appears to be C8 of G, and the imine bond was hydrolyzed to leave a salicylaldehyde end product due to the EDTA quench. A higher, faint band visible in lane 6 of Fig. 6 was also analyzed by ESI-MS and found to correspond to the intact nickel complex 8 adducted to the guanidinohydantoin moiety of the DNA strand (*i. e.* DNA + 8 - 10 = 4089 Daltons). This band is more pronounced if EDTA is omitted from the quenching step. On the salen-ArgHis ligand, the positions *ortho* and *para* to the phenol oxygen are suspected sites of reactivity based on inhibition of cross-linking when these sites are substituted in 9 (Muller, J. G.; Kayser, L.

A.; Paikoff, S. J.; Duarte, V.; Tang, N.; Rokita, S. E.; Burrows, C. J. *Coord. Chem. Rev.* 1999, 185-186, 761-774.) Final confirmation of the structure of the adduct awaits large-scale preparation and x-ray or NMR characterization.

In summary, the new hybrid ligand salen-XaaHis, as demonstrated in the prototype complex **8** (from Fig. 4), combines the chemical reactivity of a salen complex with the potential molecular recognition properties of a peptide. In principle, any protein or peptide containing histidine as the second residue (and not containing proline as the N-terminal residue) may now be converted to a DNA or RNA cross-linking agent. After hydrolysis of the imine linkage, one obtains a nucleic acid specifically derivatized with a salicylaldehyde moiety at the cross-link site. This unique aldehyde might be further conjugated to fluorescent or biotinylated probes for analysis of peptide/protein binding sites in DNA or RNA.

Example 4

Synthesis and characterization of (Ni(salen-ArgHis) OAc, **8**: 30.2 mg (0.17mmol) of *bis*-(salicylaldehyde)nickel(II) (synthesized in a 1:2 combination of Ni(OAc)₂ and salicylaldehyde) was added to 200 μ L of aqueous 0.5 M argininyhistidine carboxamide (purchased from BACHEM). The volume was adjusted to 20 mL with a 2:1 water:ethanol solution. The mixture was adjusted to pH 8 with 1 N KOH in EtOH and stirred at 25°C for 4 h. The undissolved, green, *bis*-(salicylaldehyde)nickel(II) was filtered from the solution. Orange microcrystals precipitated from the filtrate after cooling and were washed with small amounts of CHCl₃ to remove any remaining salicylaldehyde. A 40% yield (35.5 mg, 0.067 mmol) of product was realized. X-ray quality crystals were obtained by redissolving the orange microcrystals in 2:1 methanol:water and slow evaporation of solvent to form red needles. High resolution FAB-MS(positive ion) in CH₃OH gave $m/z=471.14$ (M+H⁺), Calcd $m/z=471.14$. ¹H NMR (*d*₆-DMSO): δ 1.68 (m, 2H, Arg-H_γ), 1.86 (s, 3H, acetate), 1.92 (m, 2H, Arg-H_β), 3.10 (d, 2H, His- H_β), 3.15 (m, 2H, Arg-H_α) 3.86 (m, 2H, His-H_α and Arg- H_α), 6.53 (t, 1H, salen-H₄ or H₅), 6.78 (d, 1H, salen H₆), 6.97 (s, 1H, His-H₄), 7.16 (t, 1H, salen-H₄ or H₅), 7.29 (d, 1H, salen-H₃), 7.71 (s, 1H, His-H₂), 7.89 (s, 1H, imine). ¹³C NMR(*d*₆-DMSO, ppm) 23.33, 28.47, (Arg-C_{β,γ}) 32.05 (His-C_β), 40.72 (Arg-C_δ), 50.17

(His-C α), 68.80 (Arg- C α), 114.17, 114.58, 120.43, 120.65, (salen-C $_3$, C $_6$ and C $_1$, His-C $_2$), 132.89, 132.97, 133.40, 134.93, (salen-C $_4$ and C $_5$, His-C $_1$ and C $_4$) 156.60 (guanidino), 161.54, 162.84, 173.98, 178.01 (imine, salen-C $_2$, and 2 amide C=O). IR bands at 1675, 1620, 1589, 1476, 1389, 1204, 834, 801, 721 and 625 cm $^{-1}$. UV-vis: 322 nm (ϵ =3860), shoulder at 342 nm (ϵ =2990), 398 nm (ϵ =1930 cm $^{-1}$ M $^{-1}$).

DNA experiments: The oligonucleotide was purchased from Oligos, etc. and purified by FPLC (pH 12, anion exchange). The DNA was 5' end-labeled with 32 P using T4 kinase and (γ - 32 P)-ATP. Each reaction contained 3 μ M (10 nCi) of oligodeoxynucleotide, 3 μ M calf thymus DNA (per base pair), 10 mM NaP $_i$ (pH 7.0) and 100 mM NaCl with a total volume of 50 μ L. The nickel complex was added in aliquots of 10 μ L and allowed to incubate with the DNA for 10 min. Then, a 2- μ L aliquot of the specified oxidant was added and the samples were left open to the air since O $_2$ is necessary to activate sulfite. After 1 hour, the samples were quenched with 2 μ L of 250 mM EDTA (pH=8) and individually dialyzed with water (3 x 12 hrs.). Piperidine treatment involved the addition of 60 μ L of 0.2 M piperidine to the dried samples and incubation for 30 min. at 90° C.

The ESI-MS samples were prepared by isolating the high molecular weight adduct on a gel and then precipitating the DNA with ethanol/isopropanol. Forty Eppendorf tubes of the reaction of 8 with DNA and sulfite were prepared. Each reaction mixture contained, 20 μ M of the oligodeoxynucleotide, 200 μ M of Ni(salen-ArgHis), 100 mM NaCl/10 mM NaP $_i$, and 1 mM sulfite in a final 100 μ L reaction volume. The reactions were left open to the air and quenched with 10 mM EDTA after 2 ½ hours. The samples were dialyzed with a 1000 dalton molecular weight cut-off membrane against water for 2 days. The reacted material was separated from starting material by gel electrophoresis using a 20% polyacrylamide denaturing gel. The bands were visualized by placing the gel on a silica plate shining UV light on it. The band corresponding to the high molecular weight adduct was cut out and deposited in a microcentrifuge tube filters (Spin-X, 0.22 μ M cellulose acetate) with 300 μ L of water. After agitating the tubes for several hours the samples were centrifuged to separate the sample from the gel and then dialyzed against water for 2 days. The samples were dialyzed for one more day against 10 mM ammonium acetate. The

samples were combined into 2 tubes and freeze dried. The samples were dissolved in 30 μ L of 10 M ammonium acetate and 30 μ L of water. Then, the high molecular weight adduct was precipitated with ethanol/isopropanol. After 30 μ L of water was added, 0.36 OD of product was obtained. This sample was freeze dried then dissolved in 50 μ L of a 1:1 solution of 1 mM HN_4OAc (pH 7) and isopropanol for injection into the mass spectrometer (Fig. 8).

Example 5 - Experimental details of (Ni(salen-Lys(biotin)HisArg) 12 synthesis

The tripeptide ligand was synthesized using a general solid phase peptide synthesis protocol. The peptide was synthesized on a Rink Amide AM resin, which after cleavage of the peptide, leaves a carboxamide C-terminus. In the first coupling to the resin, Fmoc-Arg(pbf)-OH was added. An endcapping step was added using acetic anhydride. The N-terminus was deprotected with a 50% piperidine solution in DMF. Then Fmoc-His(trt)-OH was coupled to the growing peptide chain. After the subsequent endcapping and deprotection steps, Dde-Lys(fmoc)-OH was added. The fmoc group on the α amino group was removed with piperidine and then coupled to D(+)-Biotin. The coupling steps were activated by coupling reagents N-methylmorpholine and HBTU in DMF. The Dde protecting group on the N-terminus of Lys(biotin)HisArg 11 was removed with 2% hydrazine in DMF. The peptide chain was cleaved from the resin using a cleavage cocktail of 95% TFA and 0.5% TIS. The cleavage cocktail was reacted with the resin beads for three hours using a wrist-action shaker. The cleaved peptide solution was collected and dried. The residue was washed with ether and dried. The peptide was purified by HPLC using a gradient of 0.1% TFA aqueous solution with a 60% acetonitrile/40% of aqueous 0.1% TFA solution. The peak with a retention time of 9 min was collected. ESI-MS confirmed that this peak contained the tripeptide. An NMR spectra showed that the sample was pure. The metal complex of the peptide ligand was prepared by Schiff base formation at the N-terminus and deprotonation of the amide. An aqueous 4 mM solution of Lys(biotin)HisArg 11 was adjusted to pH=8 using 0.1 N KOH (in ethanol). To this solution was added an excess of $\text{Ni}(\text{salicylaldehyde})_2(\text{H}_2\text{O})_2$ 10 an insoluble green compound. The solution was stirred for 3 hours then filtered through a cellulose acetate

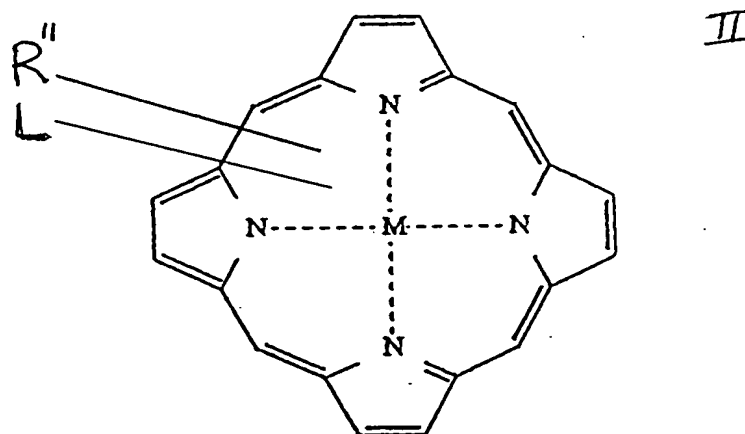
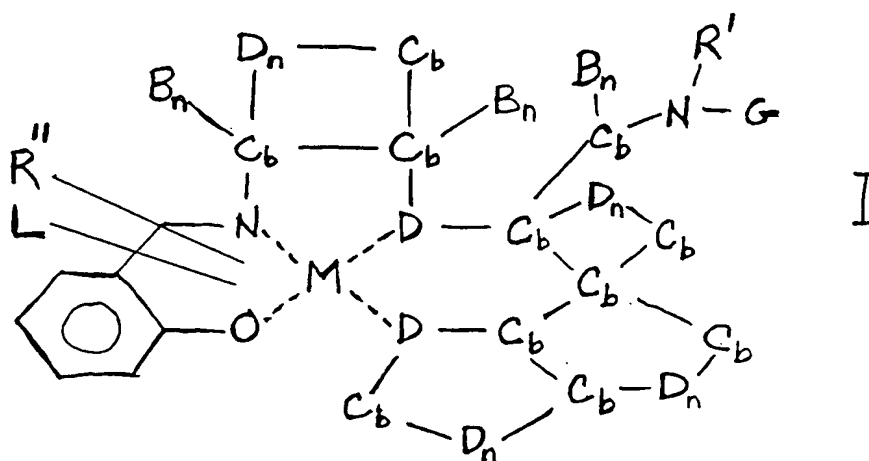
filter to remove any unreacted $\text{Ni}(\text{salicylaldehyde})_2(\text{H}_2\text{O})_2$ **10**. The solution was washed twice with an equal volume of chloroform to remove any excess salicylaldehyde. This orange solution of $[\text{Ni}(\text{salen-Lys}(\text{biotin})\text{HisArg})]^+$ **12** was shown to have the characteristic uv/vis spectrum of square planar nickel complexed to a schiff base ligand.

The protected amino acids and resin was purchased from Novasyn (Calbiochem). The Biotin was purchased from ACROS. All solvents were reagent grade.

All of the references cited herein are incorporated by reference in their entirety.

What is claimed is:

1. A compound comprising a labeled nickel complex and derivatives of Structures I-II:



wherein:

B independently represents doubly bonded oxygen;

C represents carbon;

D independently represents nitrogen, oxygen;

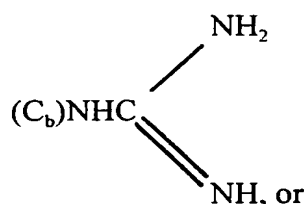
L is a detectable label, optionally attached to a linker;

M represents a nickel ion;

b=0-6;

n=0-1;

R independently represents a cationic group, optionally attached to a linker, wherein said cationic group is at least one C_b group linked to a nitrogen atom, $(CH_2)_3NH_2$, $(CH_2)_4NH_2$, $C_bN(C_b)_{0-3}$,



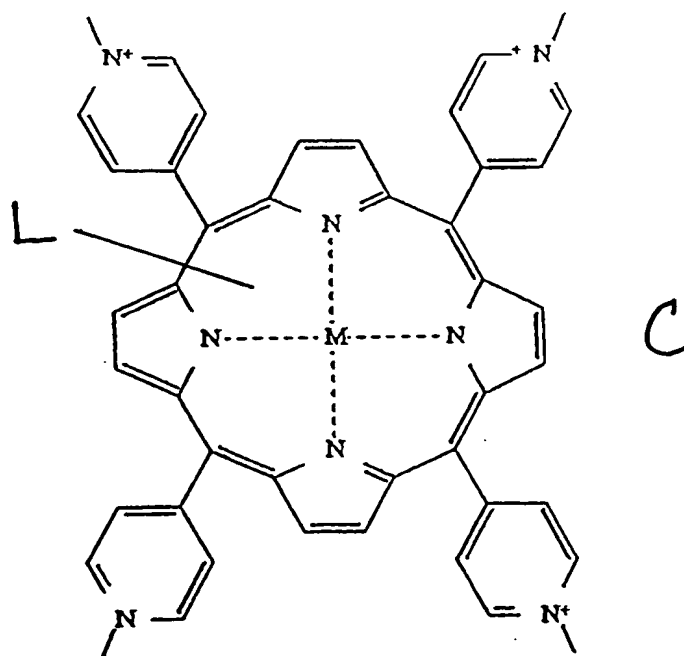
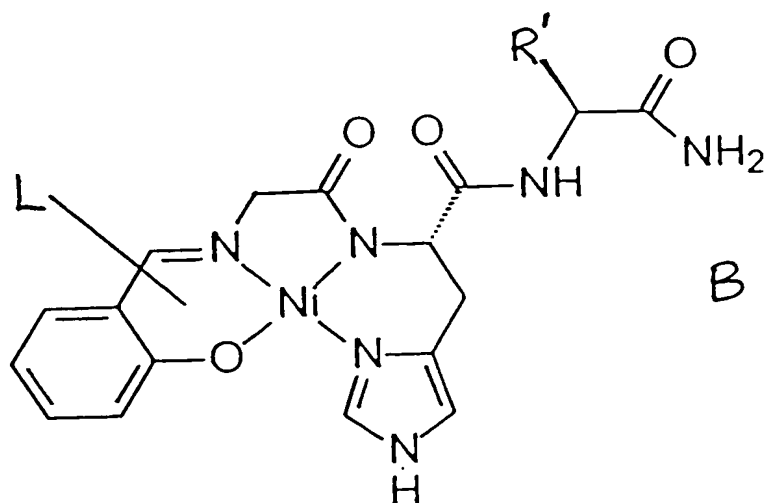
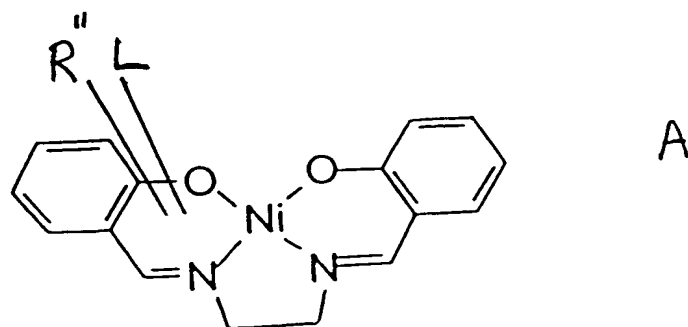
pyridyl;

R' represents hydrogen, alkyl, aryl or a continued polypeptide chain;

R'' is R or R' or G;

G represents OH, OR, a simple amide or a DNA delivery agent; and wherein all atoms contain sufficient bonds to adjacent atoms, to other atoms or to hydrogen to result in a stable structure, wherein, by independently representing is meant that within one structure, all values for the variables such as B, C, D, G, L, M, R, R', R'', b, n, need not be the same, but may represent different atoms or numbers within a single structure.

2. The compound according to claim 1, wherein said compound is structures A-C or derivatives thereof:



3. The compound according to claim 1, wherein said label is a radioactive compound, a protein ligand, a fluorescent compound, or an enzyme.
4. The compound according to claim 1, wherein said label is biotin.
5. The compound according to claim 1, wherein said nickel complex is a square planar, 4-coordinate system.
6. The compound according to claim 1, wherein said nickel complex has at least one salicylamine group, at least one cationic group, and a detectable label.
7. A method for detecting a non-canonical nucleic acid sequence comprising binding the compound according to claim 1 to a sample of nucleic acid, and detecting a signal of the detectable label on the salicylimine containing nickel complex or nickel porphyrin complex.
8. The method according to claim 7, wherein said nickel complex has at least one salicylamine group, at least one cationic group, and a detectable label.
9. The method according to claim 7, wherein said label is a radioactive compound, a protein ligand, a fluorescent compound, or an enzyme.
10. The method according to claim 9, wherein said label is biotin.
11. A labeled hybrid compound comprising the compound according to claim 1, complexed with a protein.
12. The labeled hybrid compound according to claim 11, wherein said label is a radioactive compound, a protein ligand, a fluorescent compound, or an enzyme.
13. The labeled hybrid compound according to claim 11, wherein the penultimate amino acid from the N-terminus of the protein is histidine.
14. The labeled hybrid compound according to claim 12, wherein the label is biotin.

15. The labeled hybrid compound according to claim 12, wherein the label is a flag element.

16. A method for detecting or measuring protein-nucleic acid interaction comprising mixing a labeled hybrid salicylimine or porphyrin nickel complex-protein with a solution of nucleic acid, and assaying for the signal from the detectable label attached to the nucleic acid.

17. The method according to claim 13, wherein said label is a radioactive compound, a protein ligand, a fluorescent compound, or an enzyme.

18. A method for purifying a nucleic acid-nickel-complex adduct, comprising:

mixing a labeled compound according to claim 1 with a solution of DNA,

subjecting the mixture to a separation medium, wherein the medium contains an agent that specifically binds to the label, and

separating the bound medium from the solution mixture, wherein the adduct is bound to the specific agent.

19. The method according to claim 18, wherein said separation medium is affinity chromatogram.

20. The method according to claim 19, wherein said label is biotin, and the specific agent is avidin, streptavidin or their derivatives that bind to biotin.

FIG. 1

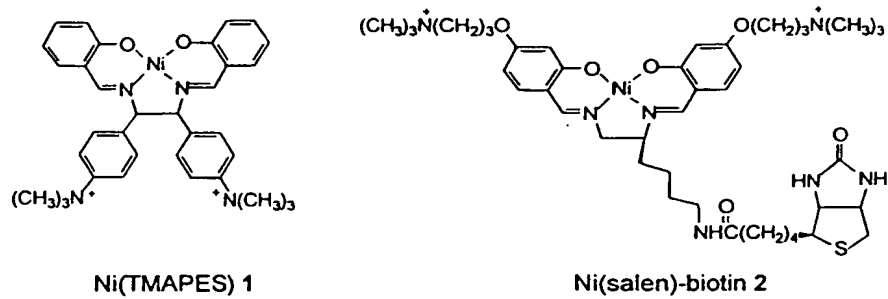
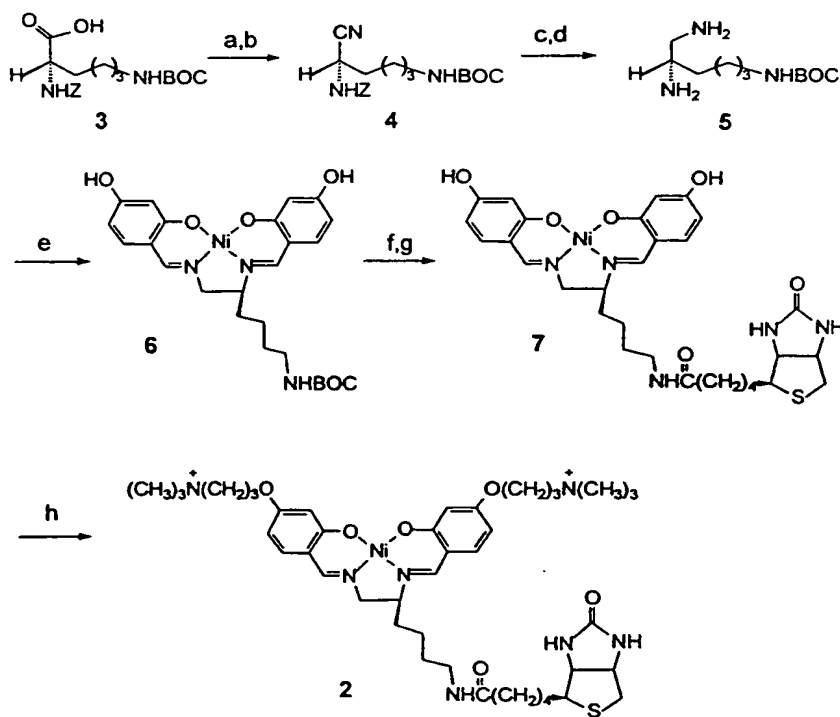


FIG. 2



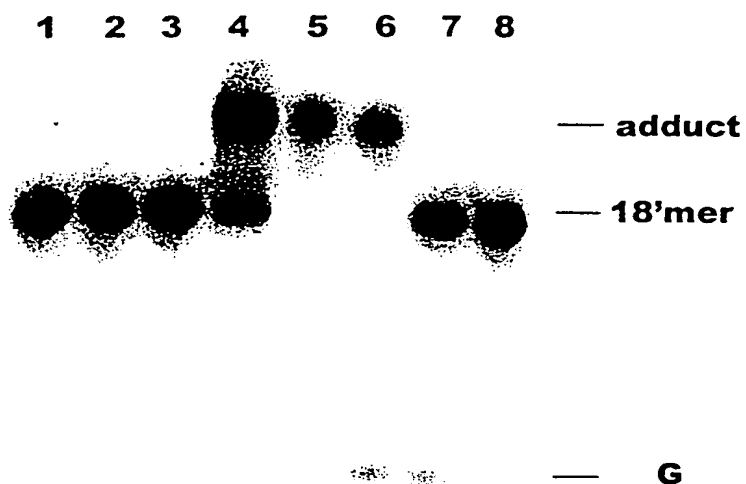


FIG. 3

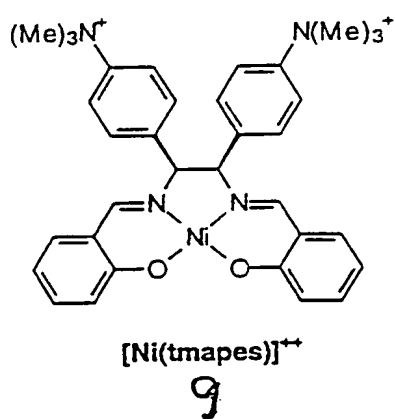
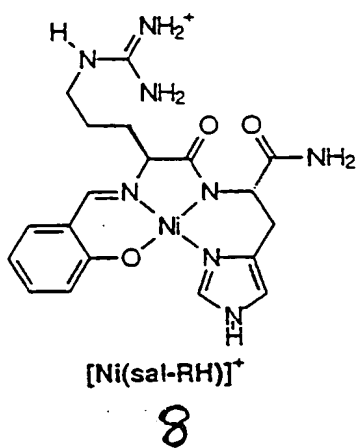


Fig. 4

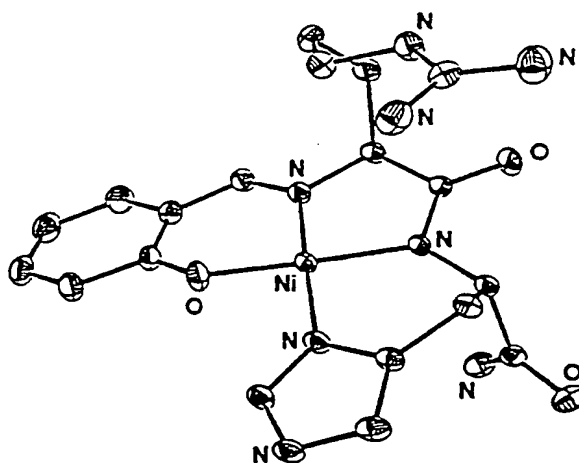


FIG. 5

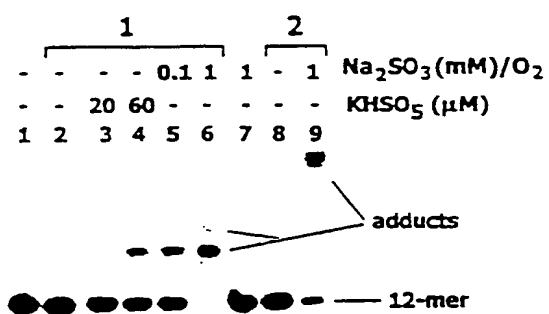
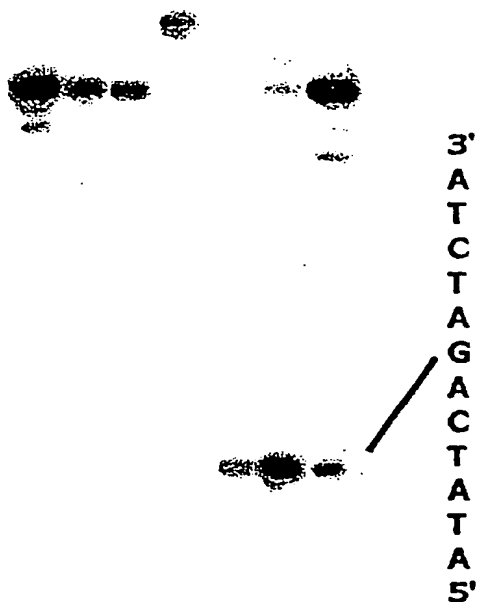
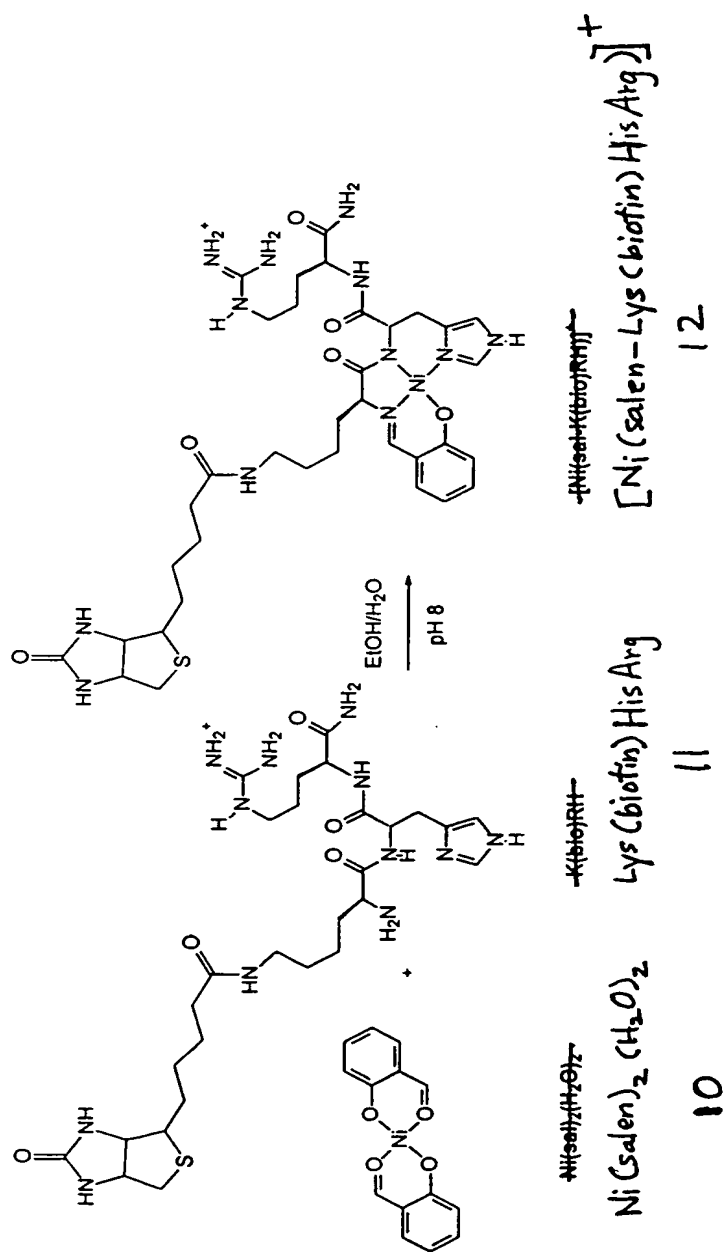


FIG. 6

FIG 7

-	-	+	-	+	+	+	piperidine
1	2	3	4	5	6	G	





9/6/14

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/18325

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : Please See Extra Sheet.

US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6; 436/86, 94; 530/300,400; 534/10, 12; 540/145; 548/106, 303.7; 556/32, 150

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WEST, CHEMICAL ABSTRACTS

search terms: Ni, nickel, porphyrin, salicylimine, salen, tmpy, nucleic, dna, rna, structures of claims 1 and 2

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,258,492 A (SCHALLY ET AL) 02 November 1993 (02/11/93), see column 4, lines 3-4, column 8, lines 42-45, 48-49, 61-63, column 27, lines 64-68.	1-3, 5, 6, 11, 12
X	US 5,268,371 A (MAUCLAIRE ET AL) 07 December 1993 (07/12/93), column 5, lines 1-27, column 8, lines 51-53, 64-68.	1-3, 5
X	US 5,272,056 A (BURROWS ET AL) 21 December 1993 (21/12/93), column 5, lines 60-67, column 6, lines 27-46, column 10, lines 34-39, column 11, lines 4-39.	1-3, 5, 6
A	US 5,595,726 A (MAGDA ET AL) 21 January 1997 (21/01/97).	1-20

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*G* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

12 OCTOBER 2000

Date of mailing of the international search report

14 NOV 2000

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/18325

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A,P	US 6,004,530 A (SAGNER ET AL) 21 December 1999 (21/12/99).	1-20
A,E	US 6,087,493 A (WHEELHOUSE ET AL) 11 July 2000 (11/07/00).	1-20
A	JP 3-38587 A (DOJINDO LABORATORIES) 19 February 1991 (19/02/91).	1-20
A	ROUTIER et al. Highly Preferential Cleavage Of Unpaired Guanines In DNA By A Functionalized Salen-Nickel Complex. Bioorganic & Medicinal Chemistry Letters. 1997, Volume 7, Number 1, pages 63-66.	1-20
A	WOODSON et al. A primer extension assay for modification of guanine by Ni(II) complexes. Nucleic Acid Research. 1993, Volume 21, Number 23, pages 5524-5525.	1-20

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/18325

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (7):

C07D 233/64, 235/02, 487/22; C07F 15/04; C07K 2/00, 14/795; G01N 33/52, 33/58, 33/60, 33/68

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/6; 436/86, 94; 530/300,400; 534/10, 12; 540/145; 548/106, 303.7; 556/32, 150

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NICKEL-BASED REAGENTS FOR DETECTING DNA AND DNA-PROTEIN CONTACTS

CONTINUING DATA

This application claims the benefit of priority to U. S. Provisional Application No. 60/141,965, filed July 1, 1999, the contents of which are incorporated herein by reference in their entirety.

GOVERNMENT INTEREST IN THE INVENTION

This invention was made with Government support under Grant Nos. GM-47531 and GM-49860 awarded by the National Institutes of Health. The Government has certain rights in this invention.

BACKGROUND OF THE INVENTION

The present invention relates to a nickel-based reagent that covalently binds to a specific nucleotide. The present invention also relates to a nickel-based salicylimine complexed to a histidine containing organic compound, as well as a nickel-based complex comprising a detectable label.

There are a variety of methods for detecting DNA/RNA-protein interactions and some depend on cross-linking. However, to the inventors' knowledge, there is no current technology that uses a nickel-based complex to covalently bind to a specific nucleotide and detect such binding and optionally isolating the DNA regions through use of a labeled complex.

A salicylimine containing nickel complex or any square planar, 4-coordinate nickel complex system linked with any detectable label is sufficient to practice the present invention. Moreover, the salicylimine containing nickel group complexed with the N-terminus of a polypeptide where the terminal amino acid is followed by a histidine or histidiny residue results in a nickel based reagent that has additional desirable functional

capabilities. If this terminal amino acid sequence is not naturally derived, then genetic manipulation may be used to append such a terminus on to any polypeptide of interest. For non-natural amino acids, chemical synthesis is preferred. Further, a semi-synthesis approach can be taken. Combining genetic and chemical means to obtain an amino acid sequence complexed with the salicylimine containing nickel complex is encompassed by the present invention. In addition, this complex may be used to add an aromatic aldehyde selectively to a nucleotide sequence which can be used as a handle for further modification or identification. Furthermore, whereas single-stranded regions of nucleic acids typically react more readily with various chemical and biochemical reagents, their detection and characterization method require prior knowledge of the target sequence. A nickel complex-identifier (such as biotin) of the invention will allow for purification and identification of the nucleic acid sequence without prior knowledge of the reactive sequence. Whereas there is no current reagent that selectively couples to and stays attached to the bound nucleotide, the nickel reagent of the invention allows for the detection of the bound sequence as well as isolation of the nucleotide region.

Shearer, J. M. and Rokita, S.E., *Bioorg. Med. Chem. Lett.* 9, 501-504, (1999), discloses the preparation of diamine compound for synthesis of a water-soluble Ni (II)-salen (salicylaldehyde) complex. This reference discloses a synthesis method for a simple, unconjugated Ni (Salen) called TMAPES. However, this reference does not disclose the attachment of a label or an adduct to this complex so that the complex can be detected biochemically.

Liang et al., *J. Am. Chem. Soc.*, 120, 248-257 (1998), discloses a Ni (II) linked metalloprotein that is used to selectively degrade DNA through a minor groove binding interaction. However, this reference does not disclose or suggest the covalent linkage between a nickel containing complex and a specific nucleotide.

Routier et al., *Bioconjugate Chem.*, 8, 789-792 (1997), discloses a conjugate between a salen and any biologically active molecule. The complex uses copper. However, such a copper conjugate cleaves nucleic acids non-specifically while non-covalently binding to the backbone of the nucleic acid. This is contrary to the present invention, which is directed to specific coupling of the nickel-containing complex to a

nucleotide base.

Routier et al., *Bioorganic & Medicinal Chemistry Letters*, Vol. 7, No. 1, pp. 63-66 (1997), discloses a Ni-Salen complex. However, in this reference, coupling the Ni-Salen complex with the terminal N group would typically create a neutral compound that would be insoluble and not useful in coupling to nucleic acid bases.

Bhattacharya and Mandal, *J. Chem. Soc., Chem. Commun.*, 1995, pp. 2489-2490, discloses a Co-Salen chemistry. Again, this reference does not disclose formation of an adduct with nucleic acid.

Tanaka et al., *Bull. Chem. Soc.*, Jpn. 70, 615-629 (1997), discloses the synthesis of a Cu-Salen molecule. This reference does not disclose or suggest formation of an adduct with nucleic acids.

Gravert and Griffin, *J. Org. Chem.*, 58, 820-822 (1993), discloses a Mn-Salen complex. This reference does not disclose the formation of an adduct with nucleic acids.

Chen and Lu, *Journal of the Chinese Chemical Society*, 45, 611-617 (1998), discloses a Ru-Salen complex. This reference does not disclose or suggest the formation of an adduct with nucleic acids.

Jacquet et al., *J. Am. Chem. Soc.* 119, 11763-11768, (1997), discloses a Ru metal - Salen complex which requires photo-activation.

Gravert and Griffin, *Inorg. Chem.*, 35, 4837-4847 (1996), discloses a Mn-Salen complex. However, this reference does not disclose the formation of an adduct with nucleic acids.

Brown et al., *Biochemistry*, 34, 4733-4739 (1995), discloses a nickel-His-tag and a nickel-glycylglycylhistidine for protein-protein cross-linking. However, the reference does not disclose nickel-dependent protein-nucleic acids or nucleic acid-biotin cross-linking.

U. S. Patent Nos. 5,272,056 and 5,504,075 disclose a Ni(TMAPES) complex but fails to disclose attaching a detectable label to this complex. These two patents are incorporated herein by reference in their entirety.

As the literature described above shows, no salicylimine containing nickel complex is known in the prior art for the application of directly detecting or isolating nucleic acids. There is a need in the art for a more effective method of studying chromosome structure,

detecting specific nucleotide bases, and studying protein-DNA interactions.

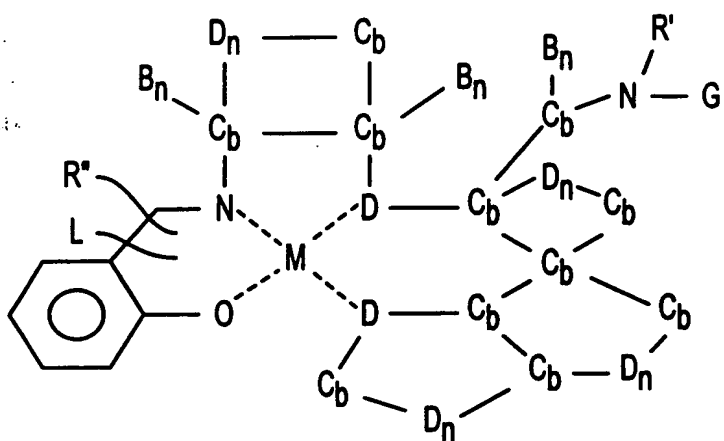
The invention provides a salicylimine containing nickel complex for detecting protein-nucleic acid interactions and the presence of single-stranded regions of nucleic acids formed during gene expression, macromolecular assembly and chromatin reorganization.

SUMMARY OF THE INVENTION

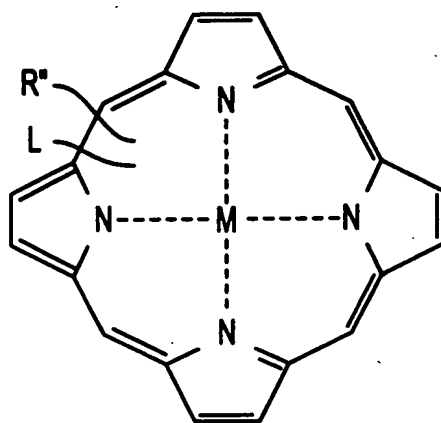
The present invention has met all of the needs stated above.

Accordingly, there is provided a process for oxidatively modifying a nucleic acid comprising the steps of providing a nucleic acid containing a target nucleotide which includes guanine, and providing a nickel-reagent that has a square planar, 4-coordinate system linked to a detectable label, which is capable of oxidative covalent coupling to the nucleic acid at or near the target sequence in the presence of an oxidizing agent. Preferably, the nickel-reagent is a salicylimine containing nickel complex, or a nickel-porphyrin complex. The nucleic acid is contacted with the salicylimine containing metal complex in the presence of an oxidizing agent so that the nucleic acid is modified at or near the target nucleotide. This also allows for the isolation of the bound nucleotide sequence region.

Structures for the nickel complex are derivatives of Structures I-II:



I



II

wherein:

B independently represents doubly bonded oxygen;

C represents carbon;

D independently represents nitrogen, oxygen;

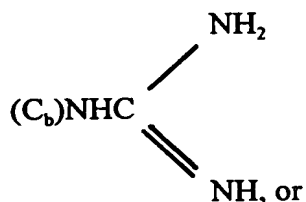
L is a detectable label, optionally attached to a linker;

M represents a nickel ion;

b=0-6;

n=0-1;

R independently represents a cationic group, optionally attached to a linker, wherein said cationic group is at least one C_b group linked to a nitrogen atom, $(CH_2)_3NH_2$, $(CH_2)_4NH_2$, $C_bN(C_b)_{0-3}$,



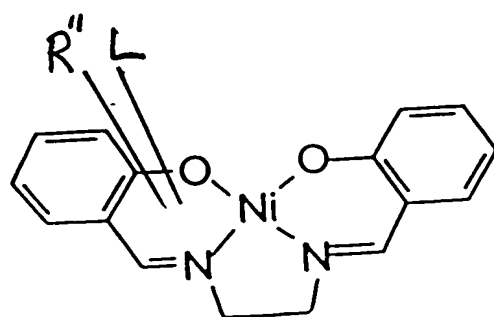
pyridyl;

R' represents hydrogen, alkyl, aryl or a continued polypeptide chain;

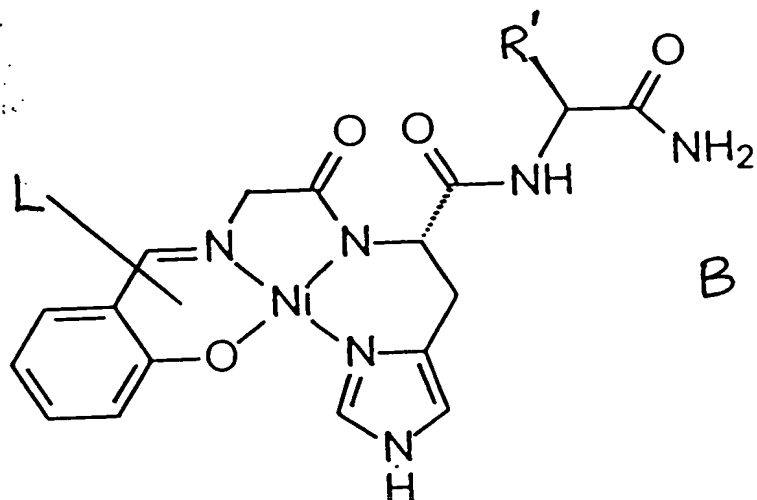
R" is R or R' or G;

G represents OH, OR, a simple amide or a DNA delivery agent; and wherein all atoms contain sufficient bonds to adjacent atoms, to other atoms or to hydrogen to result in a stable structure, wherein, by independently representing is meant that within one structure, all values for the variables such as B, C, D, G, L, M, R, R', R", b, n, need not be the same, but may represent different atoms or numbers within a single structure.

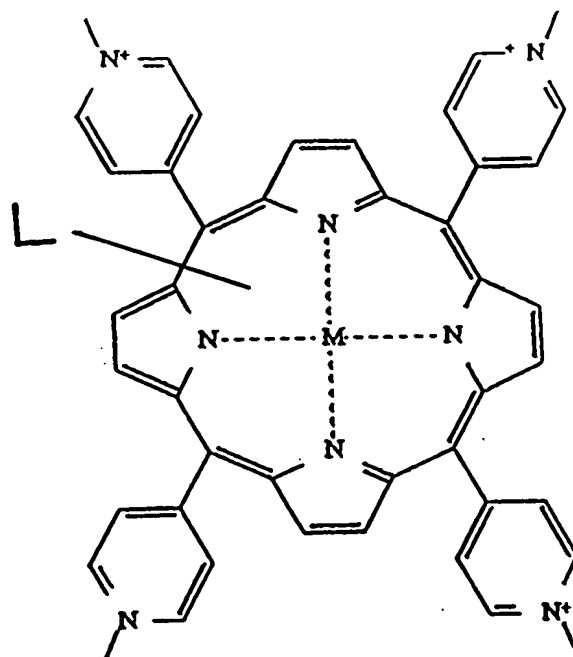
The nickel complex of Structures I-II may be structures A-C or derivatives thereof:



A



B



C

A kit is also provided for a nucleic acid assay for locating guanine groups in nucleic acids, oligonucleotides, polynucleotides, RNA and single-stranded DNA, and non-base paired guanine groups in double-stranded DNA. The kit comprises a labeled salicylimine containing metal complex selected from Structures I-II and their derivatives, an oxidant selected from the group consisting of peracid, sulfite and O₂, and optionally a base of piperidine, N-butylamine or sodium hydroxide when scission is desired.

Advantageously, the process of the invention provides a new method for detecting and isolating non-canonical DNA regions through guanine-specific modification of DNA.

Another object of the invention is to provide a nickel-based reagent to which is attached a detectable label so that the binding of the nickel-based reagent to a specific nucleotide base is detected by assaying for the label. The nucleic acid base is typically guanosine. A practical effect of the ability to detect the binding of the nickel-based reagent to the nucleic acid is the ability to isolate the bound DNA region.

It is another object of the present invention to provide a nickel-based reagent complex that is complexed with an amino acid sequence. Preferably, the amino acid sequence includes histidine. The label can be attached to either the Salen complex itself or to one of the amino acids. Preferably, Salen is complexed with an α -amino group on the N-terminal end of the amino acid sequence, and a histidine group, so that a square planar complex is formed with a nickel ion complexed with it.

Another object of the invention is to provide a reagent kit to serve industrial and academic research laboratories in research related to discerning the chromatin structure and protein-nucleic acid interaction.

These and other objects of the invention will be more fully understood from the following description of the invention, the referenced drawings attached hereto and the claims appended hereto.

BRIEF DESCRIPTION OF THE DRAWINGS

The present invention will become more fully understood from the detailed description given hereinbelow, and the accompanying drawings which are given by way of illustration only, and thus are not limitative of the present invention, and wherein;

FIGURE 1 shows Ni(salen) probes for nucleic acid structure.

FIGURE 2 shows synthesis of a Ni(salen)-biotin conjugate with solubility in water.

Reagents and conditions (a), Et_3N , ClCOOEt , NH_3 , THF, 10°C -rt, 56 %. (b) $(\text{CF}_3\text{CO})_2\text{O}$, Et_3N , THF, N_2 , 0°C , 87 %. (c) H_2 , Ra-Ni, 6.8 atm, NH_3 , MeOH, 3 days, 72 %. (d) H_2 , Pd/C, MeOH, rt, overnight, 82 %. (e) 2, 4-dihydroxy-benzaldehyde, NiAc_2 , EtOH, reflux, N_2 , 3hr, 78 %. (f) TFA, CH_2Cl_2 , rt, 0.5 h, 86 %. (g) BNHS, Et_3N , DMF, 4°C , 3 days, 22 %. (h) $\text{Br}(\text{CH}_2)_3\text{N}(\text{CH}_3)_3\text{Br}$, DMF, rt, 3days, 80 %.

FIGURE 3 shows a phosphorimage of a denaturing polyacrylamide gel (20%, 7 M urea) used to identify the high-molecular weight conjugate formed between DNA and Ni(salen)-biotin. The 5'- ^{32}P labeled oligodeoxynucleotide (18' mer), 5'-d(AAAATATCAGATCTAAAA) (12 mM, 6 nCi), in 10 mM sodium phosphate pH 7 (lane 1) was alternatively incubated with Ni(salen)-biotin 2 (50 mM, lane 2), MMPP (120 mM, lane 3) and their combination (lane 4). The resulting DNA conjugate was isolated from a monomeric avidin affinity column (lane 5) and subsequently treated with piperidine (0.2 M, 90°C , 30 min, lane 6). A standard G-lane was generated by dimethylsulfate as described by Maxam and Gilbert (lane 7) (Maxam, A. M.; Gilbert, W. *Methods Enzymol.* 1980, 65, 499-560). As a control, the parent oligonucleotide was also treated with piperidine (0.2 M, 90°C , 30 min, lane 8).

FIGURE 4 shows the formula for $(\text{Ni}(\text{salen-ArgHis}))^+$.

FIGURE 5 shows an ORTEP plot of $[\text{Ni}(\text{salen-ArgHis})]\text{CH}_3\text{CO}_2 \cdot 3\text{H}_2\text{O}$, 8; the hydrogen atoms are omitted for clarity. Selected bond lengths (\AA) and angles (deg): Ni-O(1), 1.8555(14); Ni-N(1), 1.8576(14); Ni-N(2), 1.8721(15); Ni-N(3), 1.8931(14); N(1)-C(7), 1.285(3); N(1)-C(8), 1.484(2); N(2)-C(9), 1.337(2); N(2)-C(10), 1.459(2); N(5)-C(15), 1.327(3); O(1)-Ni-N(1), 94.05(7); O(1)-Ni-N(2), 177.08(8); N(1)-Ni-N(2), 85.14(7); O(1)-Ni-N(3), 87.91(7); N(1)-Ni-N(3), 177.54(7); N(2)-Ni-N(3), 92.98(7).

FIGURE 6 shows phosphorimage of 20% polyacrylamide denaturing gel (7 M urea) showing high-molecular weight adducts to DNA formed with nickel complexes. The 12-mer oligodeoxynucleotide, 5'-d(ATATCAGATCTA)-3', was labeled at the 5' terminus with ^{32}P and incubated with reactants under the standard conditions described in Supporting Information. Lane 1: DNA alone. Lane 2: control with 100 μM Ni(salen- ArgHis) only. Lanes 3-6: 100 μM 8 plus the indicated concentrations of oxidant. Lane 7: control showing the effect of Na_2SO_3 without metal complex. Lane 8: control with 2 μM 9. Lane 9: 2 μM 9 plus Na_2SO_3 . All experiments were carried out in the presence of air and included EDTA in the workup. Cross-linking yields in lanes 3, 4, 5, and 6 were 16, 36, 39, and 80%, respectively.

FIGURE 7 shows the effect of piperidine treatment of the DNA-Ni(salen-ArgHis) adduct. Reactions were run in a similar manner as those in FIGURE 6. Lane 1: control using 100 μM Ni(OAc) $_2$, 100 μM ArgHis dipeptide and 1 mM Na_2SO_3 , showing that a high-molecular-weight species does not form in the absence of salicylaldehyde. Lanes 2-6: Reactions with 100 μM 1 and 1mM Na_2SO_3 followed by piperidine treatment (0.2M, 90°C, 30 min) when so indicated. Lanes 2 and 3: 12-mer oligonucleotide band cut and isolated from a previous gel after the adduct had formed. Lanes 4 and 5: Band corresponding to high-molecular-weight adduct cut and isolated from a previous gel. Lane 6: Typical reaction of 100 μM 1, 1 mM Na_2SO_3 with piperidine treatment. Lane G: Maxam-Gilbert G lane using dimethylsulfate.

FIGURE 8 shows ESI-MS spectrum of the high molecular weight adduct isolated from the reaction of the 12-mer oligodeoxynucleotide and Ni(salen-ArgHis) with sulfite and O_2 . The molecular weight of the 12-mer itself is 3628 as determined by ESI-MS. (R.J. Perse, Ph.D., Dissertation, University of Utah, 1997) The proposed structure of the salicylaldehyde adduct is shown.

FIGURE 9 shows a schematic for synthesizing Ni(salen-Lys(biotin)HisArg) 12.

DETAILED DESCRIPTION OF THE INVENTION

The inventors have developed a series of labeled nickel-based complex that are square planar and 4-coordinate systems. Salicylimine containing nickel-based complexes to detect protein-DNA (RNA) interactions have been exemplified in the instant application. One of the advantages of the invention is that by using the complex of the present invention, regulatory regions of DNA can be identified and characterized. Furthermore, chromatin structure abnormalities can also be examined, and epigenetic modifications of chromosomes can be determined.

In a preferred embodiment, a model peptide-salicylaldehyde complex was shown to exhibit the predicted ability to cross-link to DNA in the presence of nickel and an oxidant such as sulfite. This cross-link can either be maintained or further modified to leave only an aromatic aldehyde attached to the site of modification for additional conjugation to standard detection and purification appendages. This technology is now extended to full proteins and may also be elaborated for detection through standard protocols involving biotin, digoxigenin or a flag sequence recognition molecule, such as green fluorescent protein or a specific epitope. Furthermore, a related salicylaldehyde complex has been linked to biotin for detecting single-stranded regions of DNA. This compound has been synthesized and has been tested with DNA.

It is to be understood that the invention is not limited to the particular nickel complex described above. At a minimum, the complex requires that at least the complex take a square planar, 4-coordinate system. Preferably, the complex may contain a salicylimine containing component, preferably salicylaldehyde, and one or more additional component serving as a ligand to generate a square planar complex with nickel. In addition to the salicylimine containing group, the nickel complex may be complexed with porphyrin to provide a square-planar, 4-coordinate system. Additional components could be formed by linking other natural or non-natural organic compounds such as amino acids to the complex. The complex should also be water-soluble, by for example, the presence of at least one positive charge on the complex in water. In addition, the complex can be

conjugated to recognize elements to facilitate cellular or biomolecular localization in addition to purification and identification again through standard protocols involving biotin, flag sequence, antibodies and so on.

Abbreviations

Rink Amide AM resin	4-(2',4'-dimethoxyphenyl-Fmoc-aminomethyl-phenoxyacetamido-norleucylaminomethyl resin
Fmoc	Fluorenylmethoxycarbonyl
Pbf	2,2,4,6,7-pentmethyldihydrobenzofuran-5-sulfonyl
Trt	trityl
Dde	1-(4,4-dimethyl-2,6-dioxo-cyclohexylidene)ethyl
HBTU	2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
DMF	dimethylformamide
TIS	triisopropylsilane
TFA	trifluoroacetic acid

Abbreviations for amino acids used herein are conventionally defined as described hereinbelow unless otherwise indicated.

<u>Amino Acid</u>	<u>Three-letter abbreviation</u>	<u>One-letter symbol</u>
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Asparagine or aspartic acid	Asx	B
Cysteine	Cys	C
Diaminopropionic acid	Dpr	

Glutamine	Gln	Q
Glutamine acid	Glu	E
Glutamine or glutamic acid	Glx	Z
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Ornithine	Om	
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V
Unknown	Xaa	X

Salicylimine Containing Compounds

The compounds useful in the invention are characterized in one regard by their ability to form stable complexes with transition metals. Stable complexes are those having measurable lifetimes at room temperature in water or common organic solvents.

The compounds comprise at least one ligand and a transition metal. A ligand is defined herein as a molecule that is attached to the central metal atom of a coordination

compound. Preferred ligands are tetradentate or pentadentate and may comprise either macrocyclic or non-macrocyclic molecules. Other ligands, however, can also be used. It is to be understood that a tetradentate ligand has four donor atoms while a pentadentate ligand has five donor atoms.

The donor atoms may be nitrogen or oxygen. Preferably, at least two donor atoms are nitrogens, which are separated by from two to seven carbon atoms.

Suitable nitrogen donor groups are the amino group of peptides, or amines, imines, pyridines, imidazoles, pyrroles, and pyrazoles, with imine and pyridine groups preferred. The configuration may be square planar or pyramidal, with square planar preferred, but is not limited to these. Suitable oxygen donor groups are phenol, alcohol, carboxylic acid, and carbonyl. Examples of molecules containing oxygen donor groups are salens and salophens.

The ability of the ligands to form stable complexes results from the relative positions of the donor groups. Much of the rest of the ligand consists of carbon atoms that may be thought of as collectively forming a scaffold for maintaining the proper position of the donor groups.

Substituents on the atoms of the ligand affect the properties of the compounds, such as their ability to bind the nucleotides and DNA, their effectiveness in participating in the modification of nucleotides and DNA, their solubility in various solvents, and the stability of the complex they form with transition metals. The atoms of the ligands are normally substituted with sufficient hydrogen atoms to form a stable compound. It should be appreciated, however, that any positions in the ligands, whether or not so indicated herein, may be substituted with any other group and still do substantially the same thing in substantially the same way to accomplish the same result and are, therefore, to be considered equivalent to positions bearing hydrogen atoms as substituents for the purpose of determining the scope of the present invention.

Other suitable ligands are porphyrins such as structure C.

Some of the positions shown in Structures I-II and Structures A-C do not appear to be substituents other than hydrogen. Nevertheless, these positions may be substituted by any organic or inorganic group without significantly affecting the ability of the compound

to form a complex with transition metals.

Accordingly, any one or more of these positions may be substituted by an inorganic substituent, such as a doubly bonded oxygen, i.e., carbonyl, or a singly bonded oxygen i.e., hydroxy. Some additional inorganic groups include, for example, amino, thio, halo, i.e., F, Cl, Br, and I, etc.

Organic substituents include, for example, alkyl, aryl, alkylaryl and arylalkyl. The alkyl groups may be branched or unbranched and contain 20 carbon atoms or less, preferably 8 carbon atoms or less, and more preferably 4 carbon atoms or less. Some typical examples of alkyl groups include methyl, ethyl, n-propyl, isopropyl, n-butyl, sec-butyl, tert-butyl, isobutyl, and octyl. The alkyl groups may, in whole or in part, be in the form of rings such as cyclopentyl, cyclohexyl, cycloheptyl and cyclohexylmethyl. The cyclic groups described above may be further substituted with inorganic, alkyl, or aryl groups. Any of the alkyl groups described above may have one or more double or triple bond. Moreover, any of the carbon atoms of the alkyl groups may be separated from each other or from the ring nucleus with groups such as carbonyl, oxycarbonyl, oxy, amino, thio, etc. Alkyl groups may also terminate with groups such as halo, hydroxy, amino, carboxy, etc.

Aryl substituents are typically phenyl, but may also be any other aryl groups such as, for example, pyrrolyl, furanyl, pyridyl, thiazolyl, etc. The aryl group may, further, be substituted by an inorganic, alkyl, or other aryl group. The alkylaryl and arylalkyl groups may be any combination of alkyl and aryl groups. These groups may be further substituted.

Particularly important substituent groups which may be present on the ligands are appended groups which are capable of binding to DNA or nucleotides. These will be called delivery agents. Suitable delivery agents may be of different types including intercalators, groove-binding agents, oligonucleotides, proteins or protein fragments, and polyamines. The intercalators include ethidium, methidium, acridine, proflavin, phenanthroline, etc. The groove-binding agents include distamycin and netropsin. A distamycin derivative was used to direct nucleotide scission by Schultz, P. G., et al., "Design and Synthesis of a Sequence-Specific DNA Cleaving Molecule. (Distamycin-EDTA) iron(II)", J. Am. Chem. Soc. 104, 6861-6863 (1982). Oligonucleotides may bind producing double helical or triple

helical areas. Proteins or protein fragments which bind DNA include Hin recombinase (Mack, D. P. and Dervan, P. B., "Nickel-Mediated Sequence-Specific Oxidative Cleavage of DNA by a Designed Metalloprotein", J. Am. Chem. Soc. 112, 4604-4606 (1990)). The useful polyamines include spermine and spermidine described by K. D. Stewart, "The Effect of Structural Changes in a Polyamine Backbone on its DNA-Binding Properties", Biochem, Biophys. Res. Comm. 152, 1441-1446 (1988).

The Labeled Complexes

Ligands to be used in the invention form stable complexes with transition metal ions. For the purpose of this specification, transition metals are to be understood as including metals having partly filled d or f shells in any of their commonly occurring oxidation states. The useful transition metals include ions of Ni, Co, Cu, Rh, Pd, Ir, Pt, Cr, Mn, Fe, Ru, and Os. Preferred are ions of Ni, Co and Pd. Most preferred is Ni.

The complexes are formed by contacting a salt of the metal ion with the ligand in a suitable solvent, for example, water and methanol. Progress of the complexation is easily followed visually or spectrophotometrically. Depending upon reaction conditions such as the ligand, the metal, the pH, and the solvent, the complexation reaction may occur rapidly at room temperature, or may require heating.

The complexes may be four, five or six coordinate with four preferred and square planar most preferred.

The complexes may be deprotonated, but an overall positive charge is preferred, and a highly positive charge is most preferred. Balancing anions, counterions, or salts may be as known to those skilled in the art, such as derivatives of salts e.g. perchlorate, acetate, nitrate, chlorides, bromides, iodides, and tetrachlorozincate (ZnCl_4^{2-}).

The ligand-metal complexes are used in the process of the invention to modify oligonucleotides, single-stranded DNA, non-classical duplex structures and double-stranded DNA having particular conformations, that is, extra-helical areas, cruciform DNA, abasic (non base paired G's) areas, unpaired ends, and telomeres (ends of chromosomes). In double stranded DNA, the modification is specific to these conformational areas. The oligonucleotides may be naturally occurring or synthetic. The

single-stranded DNA may also be derived from separation of duplex DNA. All forms of RNA are targets for reaction also.

A non-diffusible species is important for site-specificity in the target nucleotide. In contrast, certain complexes, e.g. Fe(II)-EDTA plus ligand, generate hydroxyl radicals which react with DNA non-specifically. In this type of non-preferred complex, the complex attaches to a binding site and generates a reactive species which diffuses with a loss of specificity. In the system described herein, the metal complex binds to a particular DNA site and the reactive agent is produced at that site when oxidant is present. A non-diffusible species is one which reacts with DNA before it is released from the specific binding site.

The complexes are used to modify or optionally nick DNA or nucleotides when used with an oxidant. Some examples of oxidizing agents include peracids such as peroxymonosulfate salts, e.g. potassium peroxymonosulfate (KHSO_5) which is commercially available under the trademark OXONE, magnesium monoperoxyphthalate (MMPP), and oxygen in combination with sulfite. Particularly preferred are MMPP or O_2 and sulfite. In physiological systems, a combination of oxygen and sulfite is preferred.

After oxidation, excess oxidant may be quenched with a high concentration of reductant such as sodium sulfite, and base treatment may be used for cleavage. Other quenching agents may include 10 mM EDTA or β -mercaptoethanol. Optionally, if scission of nucleic acid is desired, suitable bases are piperidine, N-butylamine and sodium hydroxide with a pH of about 9-13.

Reaction conditions include a temperature from about zero to about 100°C , from about 20°C to about 40°C preferred and 25°C - 37°C most preferred. Reaction time is at least about 30 seconds, from about 10 minutes to an hour preferred and from about 15 minutes to about 30 minutes most preferred. The reaction may be left for an extended period of time, e.g. up to 48 hours without adverse effects. The pH may be about 3-10, with 6.5-7.5 preferred.

For a reaction involving, for example, a nucleotide concentration of about 0.1 to about 50 picomoles of labelled 5'-ends, the metal complex may be used in an amount of from about 0.1 μM to about 10 mM, with from about 0.5 μM to about 100 μM preferred

and from about 1 μM to about 10 μM most preferred. The oxidant may be used in an amount of from about 0.1 μM to about 10 mM with about 50 μM to about 100 μM preferred. The ratio of metal complex to oxidant may range from about 99:1 to about 1:99. The oxidant is preferably in excess of the metal complex with a ratio of metal complex:oxidant ranging up to about 1:10,000, with from about 1:1 to about 1:50 preferred and 1:1 to 1:2 most preferred.

In vitro, the nucleotide or DNA is labelled in any suitable manner such as with radiolabel, and standard electrophoresis and autoradiography may be used to determine the level of modification.

The process of the invention for modifying DNA or sequencing is suitable for being attained using a kit. The kit may comprise a salicylimine containing metal complex to be used with an oxidant and optionally a base. The kit can supply a salicylimine containing metal complex in an amount of from about 0.01 to about 500 μM metal complex, preferably from about 0.1 to about 50 μM metal complex and most preferably about 10 to about 50 μM metal complex. An oxidant is selected from the group consisting of peracid, sulfite and O_2 . The preferred oxidants are oxone, MMPP, or a combination of sulfite and oxygen. The most preferred oxidant is MMPP. If the kit is to be used in an *in vivo* system, e.g., in tissue culture or in an animal, the oxidant is preferably provided by dissolved oxygen already present in the system, with the addition of sulfite. A base may also be provided, optionally and may be piperidine, N-butylamine or sodium hydroxide having a pH of about 9-13, if scission of the DNA is desired.

As used herein, "label" or "detectable label" refers to any molecule that is linked to the Ni-salen complex to provide a detectable signal. The label may be radioactive, chemiluminescent, a protein ligand, or an enzyme, or if a fluorescent group is used, a different fluorescent group may be used for different types of salicylimine containing nickel complexes. These fluorescent tags would have the property of having spectroscopically distinguishable emission spectra. If a protein ligand based detection method is desired, biotin or digoxigenin are examples of compounds that may be used as a detectable label. In the case of digoxigenin, enzymes linked to anti-digoxigenin antibodies may be used to detect the presence of digoxigenin. Enzyme-based detection assays include

for example, the use of peroxides and alkaline phosphatase, to obtain chromogenic substances. ELISA assays can be typically used in the practice of the instant invention.

However, it should be appreciated that the choice of a label depends merely on the preference of the person of ordinary skill in the art as the present invention can be performed with any detectable label at all.

The label can be linked anywhere on the salicylimine containing nickel complex. The label is preferably linked on the carbon backbone. More preferably, the label is linked adjacent to the salicylimine group. It should be understood that binding of the label to the complex can occur practically anywhere on the carbon backbone or a substituent of the backbone, directly or through a linker, such that a person of skill in the art would be able to attach the label to the nickel complex. Any group having a primary amine group could be a site of binding of the label. The person of skill in the art would know to attach a label to the nickel complex so that the label does not interfere with the binding of the salen group to the nucleic acid. For example, the attachment of the label should not destroy or hinder the formation of a square planar, 4-coordinate system.

As used herein, "linker" means any organic residue that serves to link the label to the nickel complex. Such a linker may be, without limitation, an alkyl group, or a compound having multiple CH_2 groups, an aryl group, an arylalkyl group, ethers, and so on. Other variations and substituents are possible, so long as the linker serves to connect the label to the nickel complex without hindering the binding of the complex to the nucleic acid.

Another object of the invention is to provide a method of purifying the nickel-complex-nucleotide adduct (covalently bound). If a biotin label is linked to the nickel complex, for example, then an avidin column may be used to bind the biotin label, and thereby purify the entire adduct, including the nucleic acid region. If another protein ligand is used as the label, then using a separation process comprising its corresponding antibody may be similarly used in an affinity chromatography procedure, for example. Other methods can be used so long as the method takes advantage of the covalent binding between the nickel-complex with a specific nucleotide, and that the nickel complex is directly or indirectly attached to a detectable label.

In another aspect of the invention, a library of metals, salicylaldehydes, oxidants and peptides may be screened to determine the best candidates for oxidative cross-linking to DNA. The salicylaldehyde-guanine adducts that have already been observed may be the subject of further mechanistic studies, with a view toward providing information relevant to tyrosine-DNA cross-linked lesions.

Another feature of the invention is that the Salen-LysHis metal-binding motif may be appended to the N terminus of peptide nucleic acids (PNA) for targeting specific sites in DNA or RNA oligonucleotide targets. Base specificity is examined, and the structures of potential C, T, and A adducts are determined using ESI-MS/MS from both oligomer and nucleoside model studies. Salicylaldehyde adducts may be further functionalized with biotin and fluorescein reagents or any other label.

With the Ni(Salen-LysHis) complex in hand as a redox active complex for oxidative coupling to DNA bases, we next sought the ideal recognition agent, or delivery vehicle, to target specific sites in nucleic acids. Peptide nucleic acid (PNA) oligomers have outstanding properties for nucleic acid binding and ease of synthesis. PNA now enjoys widespread use in biological chemistry since its initial discovery in 1991. Its applications range from genome cutting to drug delivery to *in situ* hybridization. Some of the pertinent features of PNA, include:

- PNAs obey Watson-Crick rules for base pairing and form a regular "antiparallel" duplex helical structure with DNA in that the N terminus of PNA normally binds with the 3' end of a DNA oligo. Parallel duplexes are also possible, but less stable. Crystal structures show conformations similar to B-form DNA for PNA-DNA. PNA-RNA has been analyzed by NMR spectroscopy and indicates an A-like helix for the RNA.
- PNAs have high binding affinity for DNA because of their neutral charge. A PNA-DNA duplex shows a melting temperature typically 1° C higher per base pair than a DNA-DNA duplex. The stability of PNA-RNA duplexes is even higher—about 1.5° C per base pair higher than RNA-RNA duplexes.
- A consequence of the higher T_m 's is that shorter sequences may be used to impart high stability to a duplex. Furthermore, a PNA oligomer will displace a DNA

strand from a duplex in order to bind to its target DNA.

- PNA is less forgiving of base pair mismatches than is DNA. A single mismatch typically leads to an 8-20° C lowering of the T_m in a PNA-DNA duplex. This means that high sequence selectivity is ensured.
- PNA forms triple helical structures but these are primarily restricted to polypyrimidine sequences. $(PNA)_2$ -DNA is more stable than PNA-(DNA)₂.
- PNA is readily synthesized using either fmoc or Boc methodology on solid support; both types of the 4 protected base monomers are commercially available. The methodology is completely compatible with peptide synthesis and several peptide-PNA conjugates are known.
- PNA shows high chemical stability to HF, ammonia and piperidine and remarkably high biostability in both human serum and cell extracts.
- The cellular uptake of PNAs is low although DNA/PNA chimeras are taken up by Vero cells and NIH3T3 cells at low (1 μ M) extracellular concentration.
- Applications of PNA currently include diagnostic methods for screening for genetic mutations, probes in pre-gel hybridization techniques (an alternative to Southern blotting) and agents for assisting MALDI-TOF analysis of single-nucleotide polymorphisms in the human genome.

The following examples are offered by way of illustration of the present invention, and not by way of limitation.

EXAMPLES

Example 1 - Synthesis and Activity of Ni(II)-salen-biotin complex 2

The oxidation and coordination chemistry of transition metal ions offer substantial opportunities for selective recognition and modification of nucleic acids. (Sigel, H., Sigel, A. Eds.; Marcel Dekker: *Metal Ions in Biological Systems*, Vols. 32 and 33; Dekker: New York, 1996.) (Pyle, A. M.; Barton, J. K. *Prog. Inorg. Chem.* 1990, 38, 413-475.) Certain nickel complexes in particular have demonstrated alternative abilities to oxidize and couple with highly accessible guanine residues in the presence of O₂ and sulfite (Muller, J. G.;

Hickerson, R. P.; Perez, R. J.; Burrows, C. J. *J. Am. Chem. Soc.* 1997, *119*, 1501-1506.) (Stemmler, A. J.; Burrows, C. J. *J. Am. Chem. Soc.* 1999, *121*, 6956-6957.) or peracids such as monoperoxysulfate or monoperoxyphthalate. (Burrows, C. J.; Rokita, S. E. *Acc. Chem. Res.* 1994, *27*, 295-301.) (Burrows, C. J.; Rokita, S. E. In *Metal Ions in Biological Systems*, Vol. 32; Sigel, H., Sigal, A. Eds. Dekker: New York, 1996; Chapter 18, pp. 537-560.) (Rokita, S. E.; Burrows, C. J. in *Current Protocols in Nucleic Acid Chemistry*; Beaucage, S. I., Bergstrom, D. E., Glick, G., Jones, R. A. Eds. Wiley, New York, 2000; Chapter 6.4.) (Muller, J. G.; Paikoff, S. J.; Rokita, S. E.; Burrows, C. J. *J. Inorg. Biochem.* 1994, *54*, 199-206.) (Muller, J. G.; Kayser, L. A.; Paikoff, S. J.; Duarte, V.; Tang, N.; Perez, R. J.; Rokita, S. E.; Burrows, C. J. *Coord. Chem. Rev.* 1999, *185-186*, 761-774.) Adducts formed between guanine and nickel complexes based on the salen ligand (ethylene-N-N'-bis(salicyaldimine)) strongly inhibit polynucleotide elongation catalyzed by reverse transcriptase and DNA polymerase and consequently allow for sensitive detection through primer extension assays. (Muller, J. G.; Paikoff, S. J.; Rokita, S. E.; Burrows, C. J. *J. Inorg. Biochem.* 1994, *54*, 199-206.) (Muller, J. G.; Kayser, L. A.; Paikoff, S. J.; Duarte, V.; Tang, N.; Perez, R. J.; Rokita, S. E.; Burrows, C. J. *Coord. Chem. Rev.* 1999, *185-186*, 761-774.) (Woodson, S. A.; Muller, J. G.; Burrows, C. J.; Rokita, S. E. *Nucleic Acids Res.* 1993, *21*, 5524-5526. Pan, J.; Woodson, S. A. *J. Mol. Biol.* 1998, *280*, 597-609.)

The salen ligand serves two functions by activating the redox chemistry of the bound Ni(II) and generating a ligand-centered radical for addition to guanine. (Muller, J. G.; Paikoff, S. J.; Rokita, S. E.; Burrows, C. J. *J. Inorg. Biochem.* 1994, *54*, 199-206.) (Muller, J. G.; Kayser, L. A.; Paikoff, S. J.; Duarte, V.; Tang, N.; Perez, R. J.; Rokita, S. E.; Burrows, C. J. *Coord. Chem. Rev.* 1999, *185-186*, 761-774.) (Goldsby, K. A., *J. Coord. Chem.* 1988, *19*, 83-90.) (Goldsby, K. A., Blaho, J. K.; Hoferkamp, L. A. *Polyhedron* 1989, *8*, 113-115.) Direct coordination between a transient Ni(III) intermediate and accessible N7 sites on guanine appear to enhance the specificity. (Shih, H.-C.; Kassahun, H.; Burrows, C. J.; Rokita, S. E. *Biochemistry* 1999, *38*, 15034-15042) Previous investigations have focused on the water-soluble salen complex **1**, NiTMAPES (Fig. 1), but a wide range of useful derivatives can be envisioned including a salen-peptide

hybrid. (Stemmler, A. J.; Burrows, C. J. *J. Am. Chem. Soc.* 1999, *121*, 6956-6957.) (Muller, J. G.; Paikoff, S. J.; Rokita, S. E.; Burrows, C. J. *J. Inorg. Biochem.* 1994, *54*, 199-206.) (Muller, J. G.; Kayser, L. A.; Paikoff, S. J.; Duarte, V.; Tang, N.; Perez, R. J.; Rokita, S. E.; Burrows, C. J. *Coord. Chem. Rev.* 1999, *185-186*, 761-774) (Shearer, J. M.; Rokita, S. E. *Bioorg. Med. Chem. Lett.* 1999, *9*, 510-504.) These nickel salens appear unique in their ability to couple with their targets rather than promote direct strand scission as common to the salen complexes of Mn, Co and Cu. (Muller, J. G.; Kayser, L. A.; Paikoff, S. J.; Duarte, V.; Tang, N.; Perez, R. J.; Rokita, S. E.; Burrows, C. J. *Coord. Chem. Rev.* 1999, *185-186*, 761-774.)

Although footprinting well-defined polynucleotides with NiTMAPES may be routine with piperidine cleavage or primer extension, obtaining the actual products of coupling can be an arduous task. As the heterogeneity of the system increases, even footprinting becomes difficult. This type of problem is common in molecular biology, and its solution often relies on a broad range of techniques based on biotin. Labeling, detecting and purifying any biotinylated species regardless of its complexity is made possible by the extraordinary affinity of biotin for avidin and streptavidin. (Launer, H. F.; Fraenkel-Conrat, H. *J. Biol. Chem.* 1951, *193*, 125-132.) (Green, N. M. *Biochem. J.* 1963, *89*, 585-620. Green, N. M. *Adv. Protein Chem.* 1975, *29*, 85-133.) (Diamonds, E. P.; Christopoulos, T. K. *Clin. Chem.* 1991, *37*, 625-636.) (Wilchek, M.; Edward, E. A., Eds. *Methods in Enzymol.* 1990, *184*, entire volume.) The properties of a combined Ni(salen)-biotin conjugate should then have the potential to diagnosis and isolate genomic sequences containing noncanonical structures of guanine. The first synthesis of such a conjugate and its initial characterization with a model oligonucleotide is described herein.

Early synthetic targets were designed in direct analogy to TMAPES and were expected to support a range of strategies for biotinylation. However, the instability and low reactivity of the essential intermediates precluded this scheme. These problems were avoided in a subsequent approach that relied on a triamine first developed by the laboratory of Bailly for use in construction of Cu(salen) derivatives. (Routier, S.; Bernier, J.-L.; Waring, M. J.; Colson, P.; Houssier, C.; Bailly, C. *J. Org. Chem.* 1996, *61*, 2326-2331.) (Routier, S.; Cotellet, N.; Catteau, J.-P.; Bernier, J. -L.; Waring, M. J.; Riou, J.-F.; Bailly,

C. Bioorg. Med. Chem. 1996, 4, 1185-1196.) N_α -Z- N_ϵ -BOC-L-lysine 3 was converted to its nitrile derivative 4 as described previously (Figure 2). (Routier, S.; Bernier, J.-L.; Waring, M. J.; Colson, P.; Houssier, C.; Bailly, C. *J. Org. Chem.* 1996, 61, 2326-2331.) (Routier, S.; Cotellet, N.; Catteau, J.-P.; Bernier, J. -L.; Waring, M. J.; Riou, J.-F.; Bailly, C. *Bioorg. Med. Chem.* 1996, 4, 1185-1196.) A subsequent procedure requiring hydrogenation under high pressure was avoided by use of two sequential hydrogenations in the presence of Raney nickel and then Pd/C to yield the diamine 5 (see Example 2 for experimental details). The BOC-protected salen derivative 6 was formed by condensation of this diamine under N_2 with two equivalents of 2,4-dihydroxybenzaldehyde in the presence of $Ni(OAc)_2$. Deprotection by standard conditions (dry TFA/ CH_2Cl_2) and coupling with the commercially available N-hydroxysuccinimide ester of biotin (BNHS) produced the neutral and water-insoluble derivative 7. The obligatory solubility in water was achieved through alkylation of the two free phenolic oxygens with 3-bromopropyltrimethyl ammonium bromide. (Kayser, L. A., M. S. thesis, University of Utah, 1997.) The desired perchlorate salt of 2 was precipitated by addition of $KClO_4$ and recrystallized from MeOH/ Et_2O . Data from electrospray mass spectroscopy as well as 1H - and ^{13}C -NMR were consistent with the desired product.

Incubation of a model oligonucleotide (18' mer, one central G) with the Ni(salen)-biotin conjugate 2 (50 μM) or the magnesium salt of monoperoxyphthalic acid (MMPP) (120 μM) in 10 mM sodium phosphate pH 7 generated no apparent products as detected by denaturing polyacrylamide gel electrophoresis (Figure 3, lanes 2 and 3). However, their combined presence produced a high molecular weight DNA adduct in approximately 60% yield (Figure 3, lane 4). This yield decreased to 5% when the ionic strength of the mixture was increased by addition of 100 mM NaCl. In either case, reaction remained specific for the sole guanine residue as indicated below. The biotinylated DNA was conveniently separated from its parent oligonucleotide with a monomeric avidin matrix (Pierce) and desalted with reverse-phase (C-18) chromatography (Figure 3, lane 5). Subsequent treatment with hot piperidine was investigated since this condition had previously induced a diagnostic strand scission of adducts formed by NiTMAPES 1. (Muller, J. G.; Paikoff, S. J.; Rokita, S. E.; Burrows, C. J. *J. Inorg. Biochem.* 1994, 54, 199-206.) (Muller, J. G.;

Kayser, L. A.; Paikoff, S. J.; Duarte, V.; Tang, N.; Perez, R. J.; Rokita, S. E.; Burrows, C. J. *Coord. Chem. Rev.* 1999, 185-186, 761-774.) The adduct of conjugate 2 demonstrated equivalent lability in the presence of hot piperidine. The resulting oligonucleotide fragment co-migrated with the guanine standard produced by Maxam-Gilbert sequencing (Figure 3, lanes 6 and 7, respectively). (Maxam, A. M.; Gilbert, W. *Methods Enzymol.* 1980, 65, 499-560.) As expected, the parent oligonucleotide exhibited no sensitivity to piperidine (Figure 3, lane 8).

Composition of the isolated adduct was confirmed by mass spectral analysis. Electrospray mass spectroscopy identified a major species of mass 6377 that is consistent with the oligonucleotide + Ni(salen) + oxygen. The additional oxygen was anticipated from MMPP-dependent oxidation of the biotin sulfide. Incubation of the unconjugated biotin under conditions analogous to the DNA experiments above led to formation of biotin sulfoxide as indicated by comparison to a standard produced by known reaction with H₂O₂. (Melville, D. B. *J. Biol. Chem.* 1954, 208, 495-500.) This oxidation did not significantly interfere with avidin affinity chromatography since the major determinant for recognition is the ureido group of biotin. (Weber, P. C.; Ohlendorf, D. H.; Wendoloski, J. J.; Salemme, F. R. *Science* 1989, 243, 85-88.)

The ability of the nickel complex to deliver a biotin tag to accessible guanine residues allows for the isolation and characterization of noncanonical structures in complex systems such as chromatin. Few alternatives are currently available that share both a dependable conformational specificity and an easy assimilation into the wide ranging and powerful techniques based on biotin recognition.

Example 2 - Materials and methods used in the synthesis of Ni(Salen)-Biotin Conjugates 2 depicted in Fig.2

General. ¹H and ¹³C NMR spectra were recorded on Bruker DPX 400 and 500 spectrometers. Chemical shifts are reported as δ values relative to internal standards such as CHCl₃, CH₃OH or CH₃CN. Mass spectra were determined using VG 7070E (FAB) and Micromass Quattro II triple quadrupole (electrospray) instruments. Column chromatography was performed with Merck kieselgel 60 silica (230 ASTM). CH₂Cl₂ was

distilled from calcium hydride; EtOH was distilled from Mg; DMF was distilled from MgSO₄; and TFA was distilled from P₂O₅. Other solvents and chemicals were used as received.

(S)-N'-(Benzyloxycarbonyl)-1,5-diaminopentane. (S)-1-Cyano-N1-(benzyloxycarbonyl)-N5-(*tert*-butyloxycarbonyl)-1,5-diaminopentane (Routier, S.; Bernier, J.-L.; Waring, M. J.; Colson, P.; Houssier, C.; Bailly, C. *J. Org. Chem.* **1996**, *61*, 2326-2331. (b) Routier, S.; Cotelte, N.; Catteau, J.-P.; Bernier, J. -L.; Waring, M. J.; Riou, J.-F.; Bailly, C. *Bioorg. Med. Chem.* **1996**, *4*, 1185-1196) **4** (500 mg, 1.38 mmol) and Raney-Ni (1 g) were added to ammonia saturated MeOH (20 mL) and stirred under hydrogen gas (6.8 atm) at 50°C. After 72 hrs, this mixture was filtered. Solvent was removed under reduced pressure to yield the desired product (colorless oil, 360 mg, 72%). ¹H NMR (CDCl₃) δ 1.4-1.6 (m, 16H), 1.78 (m, 2H), 2.70-3.10 (m, 4H), 4.8 (bs, 2H), 5.1 (bs, 2H), 7.31 (m, 5H). HRMS (FAB) Calcd (M+H⁺) C₁₉H₃₂N₃O₄: 366.2393, found: 366.2401.

(S)-6-(*tert*-Butyloxycarbonyl)-1,2,6-triaminohexane (5). A solution of the triamine above (350 mg, 0.96 mmol) in MeOH (100 mL) was combined with Pd/C (10%, 40 mg) and stirred under hydrogen gas (1 atm) over night at room temperature. The mixture was filtered and evaporated under reduced pressure to yield a crude pink solid (180 mg, 82%) that was used below without purification. ¹H NMR (CDCl₃) δ 1.40-1.60 (m, 15H), 1.88 (m, 2H), 2.70-3.10 (m, 4H), 4.80 (m, 4H). FAB *m/z* 232 (M+H⁺).

(S)-N, N'-Bis(3-hydroxysalicylidene)-6-(*tert*-butyloxycarbonyl)-1,2,6-triaminohexane nickel complex (6). (Routier, S.; Bernier, J.-L.; Waring, M. J.; Colson, P.; Houssier, C.; Bailly, C. *J. Org. Chem.* **1996**, *61*, 2326-2331. (b) Routier, S.; Cotelte, N.; Catteau, J.-P.; Bernier, J. -L.; Waring, M. J.; Riou, J.-F.; Bailly, C. *Bioorg. Med. Chem.* **1996**, *4*, 1185-1196) 2,4-Dihydroxybenzaldehyde (239 mg, 1.73 mmol) and **5** (200 mg, 0.866 mmol) were dissolved in dry EtOH (45 mL) and heated to reflux under N₂ for 30 min. Nickel acetate (861 mg, 3.46 mmol) was then added and heating was continued for 3 hours. During this period, the clear solution became orange. The solvent was evaporated under reduced pressure, and the remaining solid was subjected to silica chromatography using a solvent

system of CH₂Cl₂/MeOH (10/1). The desired product **6** was isolated in 78% yield (357 mg). ¹H NMR (CD₃OD) δ 1.26-1.52 (m, 13H), 1.80 (m, 2H), 2.97-3.04 (m, 3H), 3.15-3.16 (m, 1H), 3.63 (m, 1H), 6.09 (m, 2H), 6.23 (s, 2H), 7.00 (d, *J*₁ = 8.4 Hz, 1H), 7.05 (d, *J*₂ = 8.8 Hz, 1H), 7.44 (s, 1H), 7.47 (s, 1H). ¹³C NMR (CD₃OD) δ 24.2, 28.8, 30.8, 36.7, 41.1, 63.6, 69.4, 79.9, 105.4, 107.4, 107.5, 115.8, 116.1, 155.4, 135.7, 158.6, 162.1, 163.1, 164.5, 164.6, 166.7, 166.9. HRMS(FAB) Calcd (M+H⁺) C₂₅H₃₂N₃O₆Ni: 528.1644, found: 528.1628.

(S)-N, N'-Bis(3-hydroxysalicylidene)-1,2,6-triaminohexane, TFA, nickel complex. (Routier, S.; Bernier, J.-L.; Waring, M. J.; Colson, P.; Houssier, C.; Bailly, C. *J. Org. Chem.* 1996, *61*, 2326-2331. Routier, S.; Cotellet, N.; Catteau, J.-P.; Bernier, J. -L.; Waring, M. J.; Riou, J.-F.; Bailly, C. *Bioorg. Med. Chem.* 1996, *4*, 1185-1196.) TFA (75 μL) was added dropwise over 30 min to a solution of **6** (10 mg, 0.0189 mmol) in CH₂Cl₂ (2.0 mL) at room temperature under N₂. After stirring for an additional 30 min, the solvent was removed under reduced pressure. EtOH (5 mL) was twice added and evaporated to help remove residual TFA. The crude product was dissolved in MeOH and precipitated by addition of CH₂Cl₂ to yield an orange solid (6.9 mg, 86%). ¹H NMR (CD₃OD) δ 1.39 (m, 8H), 2.88 (m, 2H), 3.59 (m, 1H), 6.09 (m, 1H), 6.22 (s, 1H), 6.40 (d, *J*=8.2 Hz, 1H), 7.02 (m, 1H), 7.06 (m, 1H), 7.45 (m, 2H). ¹³C NMR (CD₃OD) δ 22.5, 26.7, 27.1, 35.1, 39.0, 62.2, 67.9, 103.9, 106.1, 106.2, 108.4, 114.3, 114.6, 134.0, 160.9, 161.8, 163.1, 165.5. HRMS (FAB) Calcd (M+H⁺-TFA) C₂₀H₂₄N₃O₄Ni: 428.1120, found: 428.1161.

(S)-N, N'-Bis(3-hydroxysalicylidene)-6-biotinyl-1,2,6-triaminohexane nickel complex (7). A mixture of the Boc-protected TFA salt above (20 mg, 0.047 mmol), N-hydroxysuccinimide biotin ester (16 mg, 0.047 mmol) and triethylamine (8 μL) in dry DMF (4 mL) was stirred at 4° C for 72 hours. Solvent was removed under reduced pressure, and the solid residue was subjected to silica gel chromatography using a solvent system of CH₂Cl₂/MeOH (5/1). The desired product **7** was isolated as an orange solid (6.4 mg, 22%). ¹H NMR (CD₃OD) δ 1.26-1.57 (m, 12H), 2.11 (m, 2H), 2.66 (m, 1H), 2.88 (m, 1H), 3.01 (m, 1H), 3.18 (m, 3H), 3.60 (m, 2H), 4.30 (m, 1H), 4.48 (m, 1H), 6.11 (m, 2H), 6.24 (s, 2H), 7.07 (m, 2H), 7.49 (s, 1H), 7.51 (s, 1H). ¹³C NMR (CD₃OD) δ 24.2, 26.9,

29.4, 29.7, 30.2, 30.8, 36.6, 36.8, 40.0, 41.1, 57.1, 61.6, 63.4, 63.7, 69.3, 105.4, 107.5, 107.6, 115.8, 116.1, 135.4, 135.7, 162.2, 163.2, 164.6, 166.7, 167.0, 176.1 HRMS (FAB) Calcd (M+H⁺) C₃₀H₃₈N₅O₆Ni: 654.1896, found: 654.1866.

(S)-N, N'-Bis(3-(3-trimethylaminopropoxy)salicylidene)-6-biotinyl-1,2,6-triaminohexane nickel complex (2). 3-Bromopropyltrimethylammonium bromide (15.9 mg, 0.061 mmol) and K₂CO₃ (8.6 mg) were added to a solution of 7 (10 mg, 0.0153 mmol) in DMF (3 mL) and stirred at room temperature for three days (Kayser, L. A., M. S. thesis, University of Utah, 1997). The solvent was removed under reduced pressure to yield a crude solid. The solid was dissolved in water (1 mL) and a solution of KClO₄ (1 M) was slowly added dropwise until the first indication of precipitation was evident. This mixture was cooled to 4° C overnight. The resulting precipitate was collected by filtration, redissolved in MeOH and precipitated with Et₂O. This procedure was repeated twice. The precipitate was isolated by filtration and recrystallized twice with MeOH/Ether. The desired product 2 was obtained as an orange solid (12.8 mg, 80%). ¹H NMR (CD₃CN) δ 1.25-1.50 (m, 18H), 1.75 (m, 1H), 2.03 (m, 1H), 2.70 (m, 1H), 2.80 (m, 1H), 3.03 (bs, 18H), 3.11 (m, 4H), 3.40 (m, 2H), 3.56 (m, 1H), 4.04 (m, 2H), 4.23 (m, 1H), 4.38 (m, 1H), 4.95 (s, 1H), 5.35 (bs, 1 H), 6.16-6.29 (m, 4H), 7.12 (m, 2H), 7.49 (s, 2H). ¹³C NMR (CD₃CN) δ 23.77, 26.54, 28.89, 28.95, 29.76, 30.35, 36.05, 36.41, 39.26, 41.20, 54.01, 56.36, 60.70, 62.42, 63.40, 64.96, 65.16, 69.00, 103.14, 103.27, 106.44, 134.86, 135.18, 161.78, 162.78, 163.74, 164.23, 173.75. ESI-MS: *m/z* 954.4 ([M⁺-ClO₄], 3%), 427.7 ([M⁺-2(ClO₄)]/2, 100%).

Biotin-S-oxide. MMPP (49 mg, 0.10 mmol) was added to a solution of biotin (10 mg, 0.041 mmol) in 10 mM phosphate pH 7.0 (1 mL) and incubated at room temperature for 30 min. After removing the solvent under reduced pressure, crude product was dissolved in a solution of glacial acetic acid (20 µL) and absolute ethanol (1 mL) and maintained at 4° C for 2 days. The crystalline product was filtered, washed and dried to yield 9.2 mg (85 %). m.p. 239-242° C ((Melville, D. B. *J. Biol. Chem.* 1954, 208, 495-500) 238-241° C).

DNA experiments. The oligonucleotide 5'-d(AAAATATCAGATCTAAAA) was prepared by automated solid-phase synthesis, purified using a NAPTM-25 column (Pharmacia Biotech) and 5' end-labeled with ³²P using T4 kinase and gamma-[³²P]-ATP. DNA (11 μM, 6 nCi) was mixed with the Ni(salen)-biotin conjugate 2 (50 μM) in sodium phosphate (10 mM, pH 7.0) and incubated under ambient conditions for 10 min. Reaction was then initiated by addition of MMPP (120 μM) to a final volume of 50 μL. After further incubation for 30 min, a loading buffer (8 M urea) was added, and the samples were directly analyzed by denaturing polyacrylamide gel electrophoresis (20%) and phosphoimager.

The DNA adduct formed by the Ni(salen)-biotin conjugate was isolated on a preparative scale using non-radiolabeled DNA. Reaction conditions were identical to those described above. Only the final reaction volume was increased to 2.5 mL. After incubation, this volume was reduced by 20% under vacuum and then applied directly to a monomeric avidin column (2 mL, Pierce). Unmodified DNA was eluted with a solution of 150 mM NaCl in 100 mM sodium phosphate pH 7.2. The DNA conjugate was eluted with 2 mM biotin in the solution above as described by the manufacturer. The salts and free biotin of the elution buffer were removed by reverse phase (C-18, Sep-Pak) chromatography using water and then 30% CH₃CN in water as solvents. The isolated conjugate was lyophilized, dissolved in ammonium acetate (10 M, 50 μL) and precipitated with ethanol/isopropanol prior to alternative radiolabeling and analysis by electrospray mass spectrometry. Strand fragmentation of the adduct was accomplished by resuspending a dried and radiolabeled sample in piperidine (0.2 M, 60 μL), heating to 90° C for 30 min and finally lyophilizing. Residual piperidine was removed by repeated cycles of resuspending (water) and drying the solid pellet.

Example 3 - Preparation of Salen-XaaHis Motif for Metal-Mediated Oxidative DNA-Peptide Cross-Linking

Metallopeptide motifs of the N-terminal sequence XaaXaaHis, wherein histidine resides in the third position of the peptide, were first described for albumins (Harford, C.; Sarkar, B. *Acc. Chem. Res.* 1997, 30, 123-130.) and later utilized as redox-active

bioconjugates for DNA cleavage (Mack, D. P.; Iverson, B. L.; Dervan, P. B. *J. Am. Chem. Soc.* 1988, *110*, 7572-7574.) (Shullenberger, D. F.; Eason, P. D.; Long, E. C. *J. Am. Chem. Soc.* 1993, *115*, 11038-11039.) (Nagaoka, M.; Hagihara, M.; Kuwahara, J.; Sugiura, Y. *J. Am. Chem. Soc.* 1994, *116*, 4085-4086.) and protein-protein cross-linking. (Brown, K. C.; Yang, S.-H.; Kodadek, T. *Biochemistry* 1995, *34*, 4733-4739.) Metallosalens are another class of tetradentate ligands that have seen applications to DNA chemistry. (Burrows, C. J.; Rokita, S. E. *Acc. Chem. Res.* 1994, *27*, 295-301.) (Morrow, J. T.; Kolasa, K. A. *Inorg. Chim. Acta* 1992, *195*, 245.) (Gravert, D. J.; Griffin, J. H. *Inorg. Chem.* 1996, *35*, 4837-4847.) (Routier, S.; Bernier, J.-L.; Catteau, J.-P.; Bailly, C. *Bioorg. Med. Chem. Lett.* 1997, *7*, 63-66.) (Routier, S.; Bernier, J.-L.; Waring, M. J.; Colson, P.; Houssier, C.; Bailly, C. *J. Org. Chem.* 1996, *61*, 2326-2331.) The juxtaposition of these two motifs leads to a Schiff-base metallopeptide hybrid that might combine the molecular recognition features of a peptide with the chemical reactivity of salicylaldimine complexes. Toward this end, we describe the synthesis and characterization of a prototypical member of this new "salen-XaaHis" ligand class and an example of DNA-peptide cross-linking.

Bidentate and tridentate Schiff-base adducts to peptides have been previously described, including complexes of a broad range of transition metals. (Sakurai, T.; Hongo, J.-I.; Nakahara, A. *Inorg. Chim. Acta* 1980, *46*, 205-210.) (Cole, B. M.; Shimizu, K. D.; Krueger, C. A.; Harrity, J. P. A.; Snapper, M. L.; Hoveyda, A. H. *Angew. Chem. Int. Ed. Engl.* 1996, *35*, 1668-1671.) These chelates have shown applications as diverse as asymmetric addition of CN^- to aldehydes, (Mori, A.; Nitta, H.; Kudo, M.; Inoue, S. *Tetrahedron Lett.* 1991, *32*, 4333-4336.) epoxides (Shimizu, K. D.; Cole, B. M.; Krueger, C. A.; Kuntz, K. W.; Snapper, M. L.; Hoveyda, A. H. *Angew. Chem. Int. Ed. Engl.* 1997, *36*, 1704-1707.) and imines. (Sigman, M. S.; Jacobsen, E. N. *J. Am. Chem. Soc.* 1988, *110*, 4901-4902.) The majority of these complexes involve tridentate chelation via the phenolate oxygen, the imine nitrogen and the first amide bond of the peptide. By including a histidine residue in position 2 of the peptide, we now extend the coordination environment to a square-planar, *tetradentate* mode while still allowing for C-terminal elaboration of the peptide. See Fig. 4, complexes 8 and 9.

Arginine (Arg) was chosen for position "Xaa" of the salen-XaaHis motif in order to

impart water solubility and some degree of DNA affinity to the complex. [Ni(salen-ArgHis)]OAc, **8** (Fig. 4), was synthesized by addition of *bis*-(salicylaldehyde)Ni to a pH 8, 1:2 ethanol/water solution of the dipeptide, Arg-His bearing a C-terminal carboxamide. Orange microcrystals were obtained from this solution after 4 h at room temperature; crystals suitable for x-ray crystallographic analysis were obtained by recrystallization from 2:1 methanol/water. The crystal structure of **8**, shown in Figure 5, depicts the tetradentate nature of the salen-ArgHis ligand as predicted. (Crystal data (293 K) for [Ni(salen-ArgHis)]OAc·3H₂O, **8**: NiC₂₁H₃₄N₈O₈, M_r=585.27, monoclinic, space group C2, $a=17.0767(3)$, $b=9.1342(3)$, $c=16.3666(5)$ Å, $\alpha=90$, $\beta=101.4810(16)$, $\gamma=90^\circ$, $V=2501.81(12)$ Å³, $Z=4$, GOF = 1.089. Final R values ($I>2\sigma I$): $R1=0.0334$, $wR2=0.0969$.) The coordination geometry about the nickel ion is square planar as evidenced by the very small deviation (0.001 Å) from the plane defined by the four ligand donor atoms. The overall charge on the molecule is positive due to the guanidino moiety of arginine; an acetate counter ion is present in a hydrogen-bonded network of 3 water molecules associated with the periphery of the ligand.

Consistent with the crystal structure, the solution structure of [Ni(salen-ArgHis)]⁺ appears to be that of a square-planar, diamagnetic species based on the NMR and uv-visible absorption spectra (d-d transition at 398 nm, $\epsilon=1,930$ cm⁻¹M⁻¹). Cyclic voltammetric studies of **8** (Fig. 4) as a 1 mM aqueous solution (pH 7, 10 mM NaP_i, 100 mM NaCl) showed a largely irreversible oxidation at $E_p \sim +0.85$ V vs. Ag/AgCl (scan rate = 100 mV s⁻¹), although some evidence for a small reduction at 0.80 V could be detected ($i_{pc}/i_{pa} \sim 0.4$). This behavior is consistent with ligand-centered oxidation due to the facile formation of a phenolate radical, as is typically observed in Ni(salen) complexes.

The ligand-centered redox chemistry of the new Ni(salen-XaaHis) complex immediately suggested that it might participate in an oxidative cross-link with DNA. We previously reported that the water-soluble salen complex, Ni(TMAPES), **9**, formed an adduct to guanine residues under oxidative conditions. (Burrows, C. J.; Rokita, S. E. *Acc. Chem. Res.* 1994, 27, 295-301.) (Muller, J. G.; Paikoff, S. J.; Rokita, S. E.; Burrows, C. J. *J. Inorg. Biochem.* 1994, 54, 199-206.) (Burrows, C. J.; Muller, J. G. *Chem. Rev.* 1998, 98, 1109-1151.) In those studies, treatment of DNA or RNA with **9** in the presence of KHSO₅,

led to the formation of a guanine adduct that could be characterized by electrospray ionization MS (ESI-MS), (Muller, J. G.; Kayser, L. A.; Paikoff, S. J.; Duarte, V.; Tang, N.; Rokita, S. E.; Burrows, C. J. *Coord. Chem. Rev.* 1999, 185-186, 761-774) primer extension assays, (Woodson, S. A.; Muller, J. G.; Burrows, C. J.; Rokita, S. E. *Nucleic Acids Res.* 1993, 21, 5524-5525.) and PAGE analysis of short oligonucleotides. (Muller, J. G.; Paikoff, S. J.; Rokita, S. E.; Burrows, C. J. *J. Inorg. Biochem.* 1994, 54, 199-206.) The participation of the phenolate moiety in **9** as part of the cross-link was confirmed through substituent effect studies, (Muller, J. G.; Kayser, L. A.; Paikoff, S. J.; Duarte, V.; Tang, N.; Rokita, S. E.; Burrows, C. J. *Coord. Chem. Rev.* 1999, 185-186, 761-774) and the modified guanine was found to be a piperidine-labile DNA cleavage site (Muller, J. G.; Paikoff, S. J.; Rokita, S. E.; Burrows, C. J. *J. Inorg. Biochem.* 1994, 54, 199-206.) (Muller, J. G.; Kayser, L. A.; Paikoff, S. J.; Duarte, V.; Tang, N.; Rokita, S. E.; Burrows, C. J. *Coord. Chem. Rev.* 1999, 185-186, 761-774.)

Studies reported herein show that the cross-linking behavior of a phenolate moiety can now be incorporated into the N-terminal motif of a peptide. As shown in Fig. 6, treatment of a 5'-[³²P]-end-labeled 12-mer oligodeoxynucleotide with **8** (100 μ M) and KHSO₅ (20 or 60 μ M, lane 3 or 4), followed by quenching with HEPES + EDTA, led to a high molecular weight band observable by PAGE. Interestingly, **8** appears to share with nickel peptides (Muller, J. G.; Hickerson, R. P.; Perez, R. J.; Burrows, C. J. *J. Am. Chem. Soc.* 1997, 119, 1501-1506.) and nickel salens the ability to catalyze HSO₅⁻ formation *in situ* via autoxidation of sulfite. Thus, cross-linking of **8** to DNA was even more effective when KHSO₅ was replaced with Na₂SO₃ (0.1 or 1 mM, lane 5 or 6). Sulfite can be used in greater concentration than HSO₅⁻, an oxidant that would lead to indiscriminant background oxidation of DNA if used in concentrations >100 μ M. Control studies confirmed that no high molecular weight band was observed if Ni²⁺ + ArgHis (lacking salicylaldehyde) was used in place of **8**. The position on the gel of the high molecular-weight bands from **8** are significantly lower than that from **9** (whose DNA cross-link is shown for comparison in Fig. 6, lane 9), suggesting that the charge and molecular weight of this adduct are different. It is noted that **8** was used in higher concentration than **9** because of its lower electrostatic attraction for DNA. Extension of the peptide to include a DNA-binding moiety should

greatly reduce the required concentration of metal complex.

Treatment of the [Ni(salen- ArgHis)]⁺-modified DNA with 0.2 M piperidine at 90° C for 30 min. led to substantial cleavage, but only at the single G residue of the oligomer (see Figure 7). Several other oligonucleotide sequences were used, and all gave the same G chemistry as described here. Control studies demonstrated that no other base modifications, such as imidazolone formation, (Cadet, J.; Berger, M.; Buchko, G. W.; Joshi, P. C.; Raoul, S.; Ravanat, J.-L. *J. Am. Chem. Soc.* 1994, *116*, 7403-7404.) were produced by these reaction conditions. The piperidine sensitivity of the DNA adduct suggests that the cross-link has likely formed to either N7 or C8 of G, since both of these sites of modification typically lead to piperidine-induced strand scission. (Burrows, C. J.; Muller, J. G. *Chem. Rev.* 1998, *98*, 1109-1151.) Indeed, arylation of guanine at both of these positions has been observed for the phenolic A-ring of estrogen. (Akanni, A.; Tabakovic, K.; Abul-Hajj, Y. J. *Chem. Res. Toxicol.* 1997, *10*, 477-481.) Mass spectral analysis provided further information on the site and identity of adduct formation; isolation of the major 8-DNA adduct from the gel and precipitation with NH₄OAc led to a sample suitable for ESI-MS. The final spectrum showed a single product of mass 3739 consistent with DNA + salicylaldehyde - 10 Daltons (see Figure 8). The overall loss of 10 mass units corresponds to the now generally observed phenomenon of further oxidation of any C8-modified guanine adduct leading to a guanidinohydantoin product after hydration and decarboxylation of C6 of the purine. (Duarte, V.; Muller, J. G.; Burrows, C. J. *Nucleic Acids Res.* 1999, *27*, 496-502.) (Poje, M.; Sokolic-Maravic, L. *Tetrahedron* 1988, *44*, 6723-6728.) (Shibutani, S.; Gentles, R. G.; Iden, C. R.; Johnson, F. J. *J. Am. Chem. Soc.* 1990, *112*, 5667-5668.) Thus, the DNA partner in cross-link formation appears to be C8 of G, and the imine bond was hydrolyzed to leave a salicylaldehyde end product due to the EDTA quench. A higher, faint band visible in lane 6 of Fig. 6 was also analyzed by ESI-MS and found to correspond to the intact nickel complex 8 adducted to the guanidinohydantoin moiety of the DNA strand (*i. e.* DNA + 8 - 10 = 4089 Daltons). This band is more pronounced if EDTA is omitted from the quenching step. On the salen-ArgHis ligand, the positions *ortho* and *para* to the phenol oxygen are suspected sites of reactivity based on inhibition of cross-linking when these sites are substituted in 9 (Muller, J. G.; Kayser, L.

A.; Paikoff, S. J.; Duarte, V.; Tang, N.; Rokita, S. E.; Burrows, C. J. *Coord. Chem. Rev.* 1999, 185-186, 761-774.) Final confirmation of the structure of the adduct awaits large-scale preparation and x-ray or NMR characterization.

In summary, the new hybrid ligand salen-XaaHis, as demonstrated in the prototype complex **8** (from Fig. 4), combines the chemical reactivity of a salen complex with the potential molecular recognition properties of a peptide. In principle, any protein or peptide containing histidine as the second residue (and not containing proline as the N-terminal residue) may now be converted to a DNA or RNA cross-linking agent. After hydrolysis of the imine linkage, one obtains a nucleic acid specifically derivatized with a salicylaldehyde moiety at the cross-link site. This unique aldehyde might be further conjugated to fluorescent or biotinylated probes for analysis of peptide/protein binding sites in DNA or RNA.

Example 4

Synthesis and characterization of (Ni(salen-ArgHis) OAc, **8**: 30.2 mg (0.17mmol) of *bis*-(salicylaldehyde)nickel(II) (synthesized in a 1:2 combination of Ni(OAc)₂ and salicylaldehyde) was added to 200 μ L of aqueous 0.5 M argininyhistidine carboxamide (purchased from BACHEM). The volume was adjusted to 20 mL with a 2:1 water:ethanol solution. The mixture was adjusted to pH 8 with 1 N KOH in EtOH and stirred at 25°C for 4 h. The undissolved, green, *bis*-(salicylaldehyde)nickel(II) was filtered from the solution. Orange microcrystals precipitated from the filtrate after cooling and were washed with small amounts of CHCl₃ to remove any remaining salicylaldehyde. A 40% yield (35.5 mg, 0.067 mmol) of product was realized. X-ray quality crystals were obtained by redissolving the orange microcrystals in 2:1 methanol:water and slow evaporation of solvent to form red needles. High resolution FAB-MS(positive ion) in CH₃OH gave $m/z=471.14$ (M+H⁺), Calcd $m/z=471.14$. ¹H NMR (d₆-DMSO): δ 1.68 (m, 2H, Arg-H_γ), 1.86 (s, 3H, acetate), 1.92 (m, 2H, Arg-H_β), 3.10 (d, 2H, His- H_β), 3.15 (m, 2H, Arg-H_α) 3.86 (m, 2H, His-H_α and Arg- H_α), 6.53 (t, 1H, salen-H₄ or H₅), 6.78 (d, 1H, salen H₆), 6.97 (s, 1H, His-H₄), 7.16 (t, 1H, salen-H₄ or H₅), 7.29 (d, 1H, salen-H₃), 7.71 (s, 1H, His-H₂), 7.89 (s, 1H, imine). ¹³C NMR(d₆-DMSO, ppm) 23.33, 28.47, (Arg-C_{β,γ}) 32.05 (His-C_β), 40.72 (Arg-C_α), 50.17

(His-C_α), 68.80 (Arg- C_α), 114.17, 114.58, 120.43, 120.65, (salen-C₃, C₆ and C₁, His-C₂), 132.89, 132.97, 133.40, 134.93, (salen-C₄ and C₅, His-C₁ and C₄) 156.60 (guanidino), 161.54, 162.84, 173.98, 178.01 (imine, salen-C₂, and 2 amide C=O). IR bands at 1675, 1620, 1589, 1476, 1389, 1204, 834, 801, 721 and 625 cm⁻¹. UV-vis: 322 nm (ε=3860), shoulder at 342 nm (ε=2990), 398 nm (ε=1930 cm⁻¹M⁻¹).

DNA experiments: The oligonucleotide was purchased from Oligos, etc. and purified by FPLC (pH 12, anion exchange). The DNA was 5' end-labeled with ³²P using T4 kinase and (γ-³²P)-ATP. Each reaction contained 3 μM (10 nCi) of oligodeoxynucleotide, 3 μM calf thymus DNA (per base pair), 10 mM NaP_i (pH 7.0) and 100 mM NaCl with a total volume of 50 μL. The nickel complex was added in aliquots of 10 μL and allowed to incubate with the DNA for 10 min. Then, a 2-μL aliquot of the specified oxidant was added and the samples were left open to the air since O₂ is necessary to activate sulfite. After 1 hour, the samples were quenched with 2 μL of 250 mM EDTA (pH=8) and individually dialyzed with water (3 x 12 hrs.). Piperidine treatment involved the addition of 60 μL of 0.2 M piperidine to the dried samples and incubation for 30 min. at 90° C.

The ESI-MS samples were prepared by isolating the high molecular weight adduct on a gel and then precipitating the DNA with ethanol/isopropanol. Forty Eppendorf tubes of the reaction of 8 with DNA and sulfite were prepared. Each reaction mixture contained, 20 μM of the oligodeoxynucleotide, 200 μM of Ni(salen-ArgHis), 100 mM NaCl/10 mM NaP_i, and 1 mM sulfite in a final 100μL reaction volume. The reactions were left open to the air and quenched with 10 mM EDTA after 2 ½ hours. The samples were dialyzed with a 1000 dalton molecular weight cut-off membrane against water for 2 days. The reacted material was separated from starting material by gel electrophoresis using a 20% polyacrylamide denaturing gel. The bands were visualized by placing the gel on a silica plate shining UV light on it. The band corresponding to the high molecular weight adduct was cut out and deposited in a microcentrifuge tube filters (Spin-X, 0.22 μM cellulose acetate) with 300 μL of water. After agitating the tubes for several hours the samples were centrifuged to separate the sample from the gel and then dialyzed against water for 2 days. The samples were dialyzed for one more day against 10 mM ammonium acetate. The

samples were combined into 2 tubes and freeze dried. The samples were dissolved in 30 μL of 10 M ammonium acetate and 30 μL of water. Then, the high molecular weight adduct was precipitated with ethanol/isopropanol. After 30 μL of water was added, 0.36 OD of product was obtained. This sample was freeze dried then dissolved in 50 μL of a 1:1 solution of 1 mM HN_4OAc (pH 7) and isopropanol for injection into the mass spectrometer (Fig. 8).

Example 5 - Experimental details of (Ni(salen-Lys(biotin)HisArg) 12 synthesis

The tripeptide ligand was synthesized using a general solid phase peptide synthesis protocol. The peptide was synthesized on a Rink Amide AM resin, which after cleavage of the peptide, leaves a carboxamide C-terminus. In the first coupling to the resin, Fmoc-Arg(pbf)-OH was added. An endcapping step was added using acetic anhydride. The N-terminus was deprotected with a 50% piperidine solution in DMF. Then Fmoc-His(trt)-OH was coupled to the growing peptide chain. After the subsequent endcapping and deprotection steps, Dde-Lys(fmoc)-OH was added. The fmoc group on the α amino group was removed with piperidine and then coupled to D(+)-Biotin. The coupling steps were activated by coupling reagents N-methylmorpholine and HBTU in DMF. The Dde protecting group on the N-terminus of Lys(biotin)HisArg 11 was removed with 2% hydrazine in DMF. The peptide chain was cleaved from the resin using a cleavage cocktail of 95% TFA and 0.5% TIS. The cleavage cocktail was reacted with the resin beads for three hours using a wrist-action shaker. The cleaved peptide solution was collected and dried. The residue was washed with ether and dried. The peptide was purified by HPLC using a gradient of 0.1% TFA aqueous solution with a 60% acetonitrile/40% of aqueous 0.1% TFA solution. The peak with a retention time of 9 min was collected. ESI-MS confirmed that this peak contained the tripeptide. An NMR spectra showed that the sample was pure. The metal complex of the peptide ligand was prepared by Schiff base formation at the N-terminus and deprotonation of the amide. An aqueous 4 mM solution of Lys(biotin)HisArg 11 was adjusted to pH=8 using 0.1 N KOH (in ethanol). To this solution was added an excess of $\text{Ni}(\text{salicylaldehyde})_2(\text{H}_2\text{O})_2$ 10 an insoluble green compound. The solution was stirred for 3 hours then filtered through a cellulose acetate

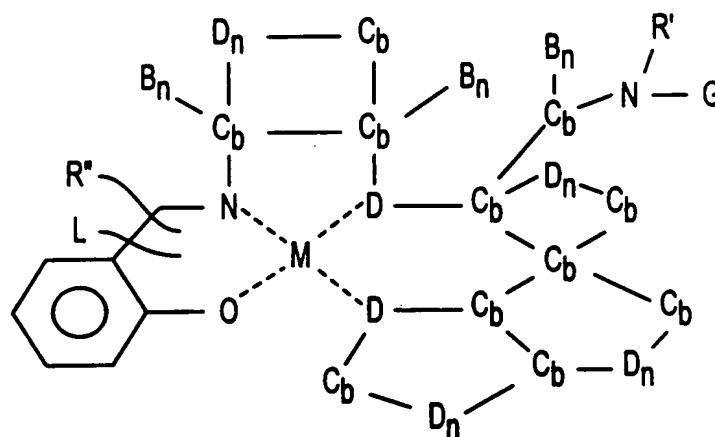
filter to remove any unreacted $\text{Ni}(\text{salicylaldehyde})_2(\text{H}_2\text{O})_2$ **10**. The solution was washed twice with an equal volume of chloroform to remove any excess salicylaldehyde. This orange solution of $[\text{Ni}(\text{salen-Lys}(\text{biotin})\text{HisArg})]^+$ **12** was shown to have the characteristic uv/vis spectrum of square planar nickel complexed to a schiff base ligand.

The protected amino acids and resin was purchased from Novasyn (Calbiochem). The Biotin was purchased from ACROS. All solvents were reagent grade.

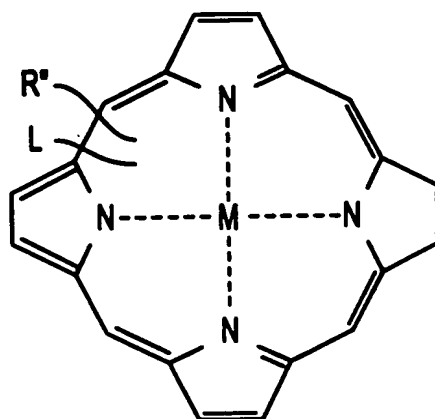
All of the references cited herein are incorporated by reference in their entirety.

What is claimed is:

1. A compound comprising a labeled nickel complex and derivatives of Structures I-II:



I



II

wherein:

B independently represents doubly bonded oxygen;

C represents carbon;

D independently represents nitrogen, oxygen;

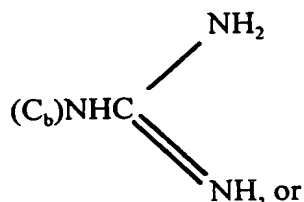
L is a detectable label, optionally attached to a linker;

M represents a nickel ion;

b=0-6;

n=0-1;

R independently represents a cationic group, optionally attached to a linker, wherein said cationic group is at least one C_b group linked to a nitrogen atom, $(CH_2)_3NH_2$, $(CH_2)_4NH_2$, $C_bN(C_b)_{0-3}$,



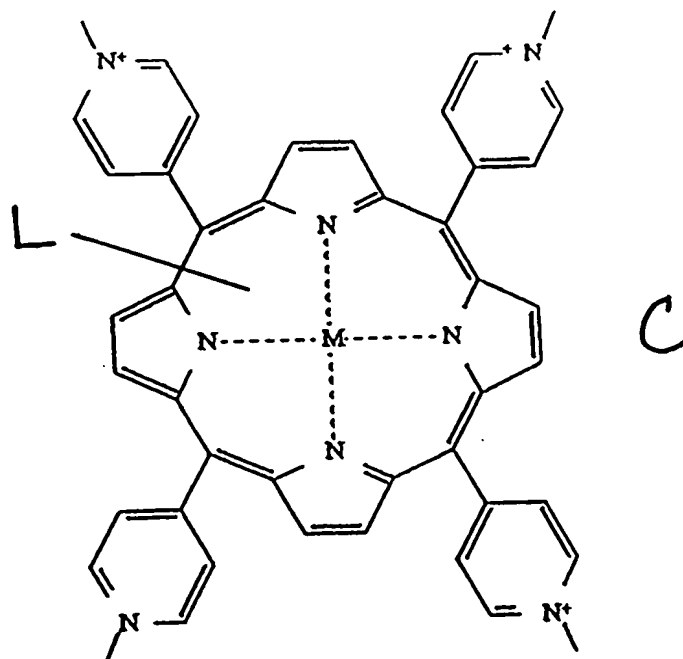
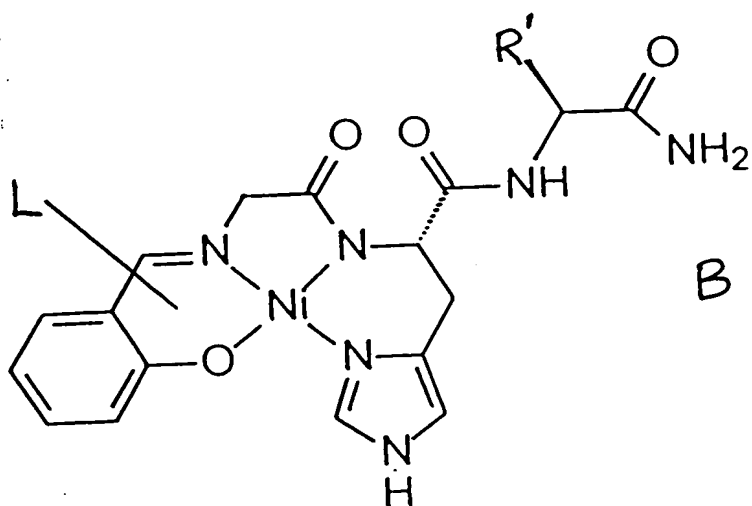
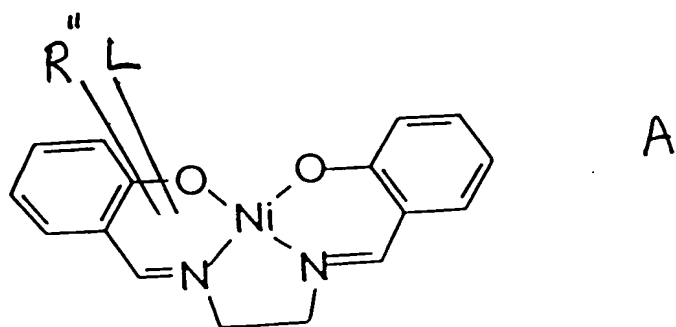
pyridyl;

R' represents hydrogen, alkyl, aryl or a continued polypeptide chain;

R" is R or R' or G;

G represents OH, OR, a simple amide or a DNA delivery agent; and wherein all atoms contain sufficient bonds to adjacent atoms, to other atoms or to hydrogen to result in a stable structure, wherein, by independently representing is meant that within one structure, all values for the variables such as B, C, D, G, L, M, R, R', R" b, n, need not be the same, but may represent different atoms or numbers within a single structure.

2. The compound according to claim 1, wherein said compound is structures A-C or derivatives thereof:



3. The compound according to claim 1, wherein said label is a radioactive compound, a protein ligand, a fluorescent compound, or an enzyme.
4. The compound according to claim 1, wherein said label is biotin.
5. The compound according to claim 1, wherein said nickel complex is a square planar, 4-coordinate system.
6. The compound according to claim 1, wherein said nickel complex has at least one salicylamine group, at least one cationic group, and a detectable label.
7. A method for detecting a non-canonical nucleic acid sequence comprising binding the compound according to claim 1 to a sample of nucleic acid, and detecting a signal of the detectable label on the salicylamine containing nickel complex or nickel porphyrin complex.
8. The method according to claim 7, wherein said nickel complex has at least one salicylamine group, at least one cationic group, and a detectable label.
9. The method according to claim 7, wherein said label is a radioactive compound, a protein ligand, a fluorescent compound, or an enzyme.
10. The method according to claim 9, wherein said label is biotin.
11. A labeled hybrid compound comprising the compound according to claim 1, complexed with a protein.
12. The labeled hybrid compound according to claim 11, wherein said label is a radioactive compound, a protein ligand, a fluorescent compound, or an enzyme.
13. The labeled hybrid compound according to claim 11, wherein the penultimate amino acid from the N-terminus of the protein is histidine.
14. The labeled hybrid compound according to claim 12, wherein the label is biotin.

15. The labeled hybrid compound according to claim 12, wherein the label is a flag element.

16. A method for detecting or measuring protein-nucleic acid interaction comprising mixing a labeled hybrid salicylimine or porphyrin nickel complex-protein with a solution of nucleic acid, and assaying for the signal from the detectable label attached to the nucleic acid.

17. The method according to claim 13, wherein said label is a radioactive compound, a protein ligand, a fluorescent compound, or an enzyme.

18. A method for purifying a nucleic acid-nickel-complex adduct, comprising:

mixing a labeled compound according to claim 1 with a solution of DNA,

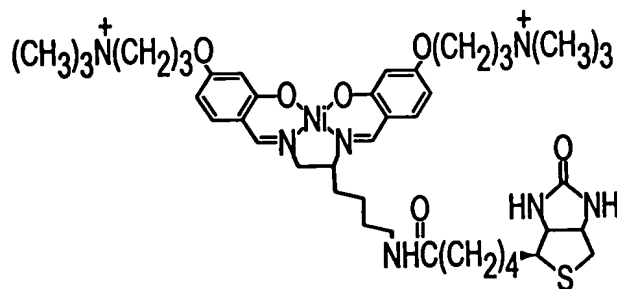
subjecting the mixture to a separation medium, wherein the medium contains an agent that specifically binds to the label, and

separating the bound medium from the solution mixture, wherein the adduct is bound to the specific agent.

19. The method according to claim 18, wherein said separation medium is affinity chromatogram.

20. The method according to claim 19, wherein said label is biotin, and the specific agent is avidin, streptavidin or their derivatives that bind to biotin.

21. A compound comprising a labeled nickel complex and derivatives of



22. A method for detecting a non-canonical nucleic acid sequence comprising binding the compound according to claim 21 to a sample of nucleic acid, and detecting a signal of the detectable label on the salicylimine containing nickel complex or nickel porphyrin complex.

23. A labeled hybrid compound comprising the compound according to claim 21, complexed with a protein.

24. A method for detecting or measuring protein-nucleic acid interaction comprising mixing the compound according to claim 23 with a solution of nucleic acid, and assaying for the signal from the detectable label attached to the nucleic acid.

25. A method for purifying a nucleic acid-nickel-complex adduct, comprising:
 mixing a labeled compound according to claim 21 with a solution of DNA,
 subjecting the mixture to a separation medium, wherein the medium contains an agent that specifically binds to the label, and
 separating the bound medium from the solution mixture, wherein the adduct is bound to the specific agent.

ABSTRACT OF THE INVENTION

The invention relates to a compound comprising a labeled nickel complex that is used to detect and purify nucleic acid sequences by forming an adduct between a specific nucleic acid base and the labeled nickel complex. The adduct can also be purified through a separation process, such as affinity chromatography.

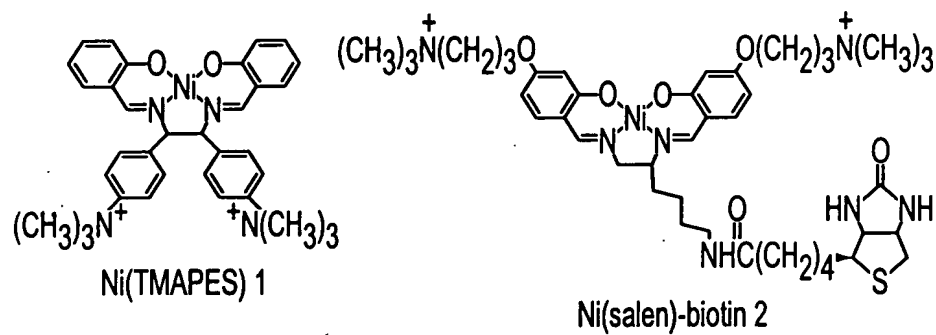


FIG. 1

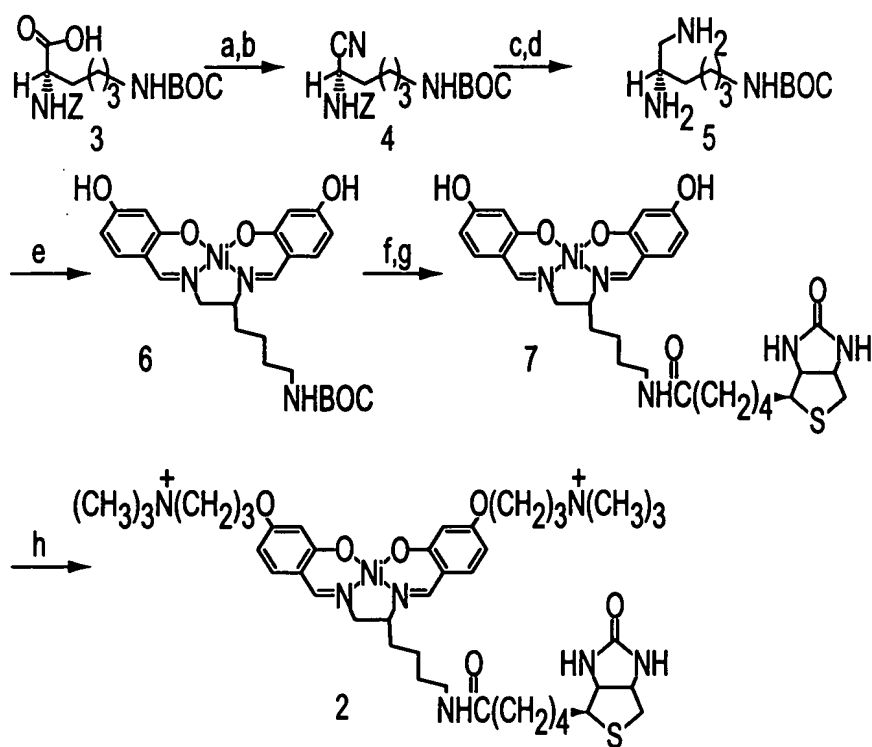


FIG. 2

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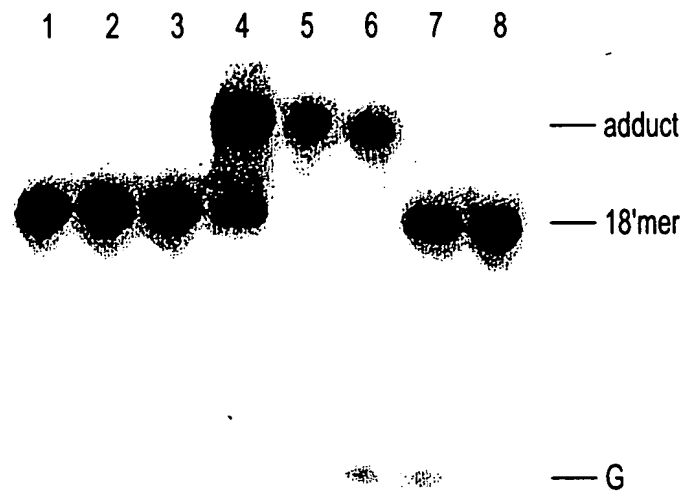


FIG. 3

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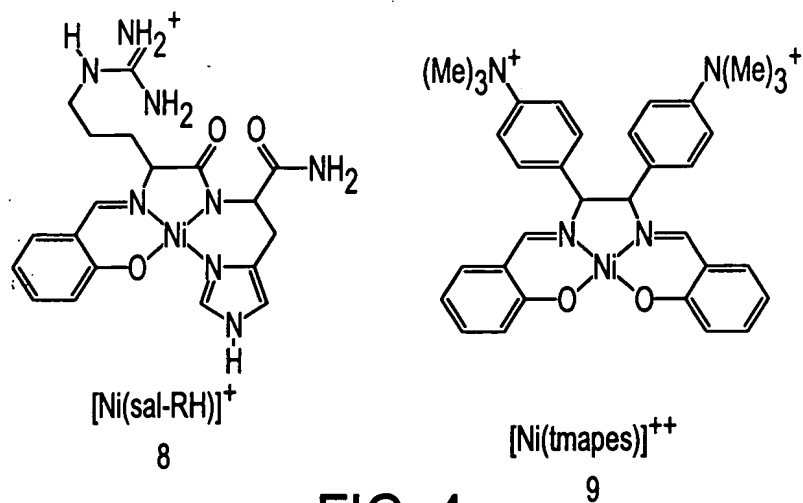


FIG. 4

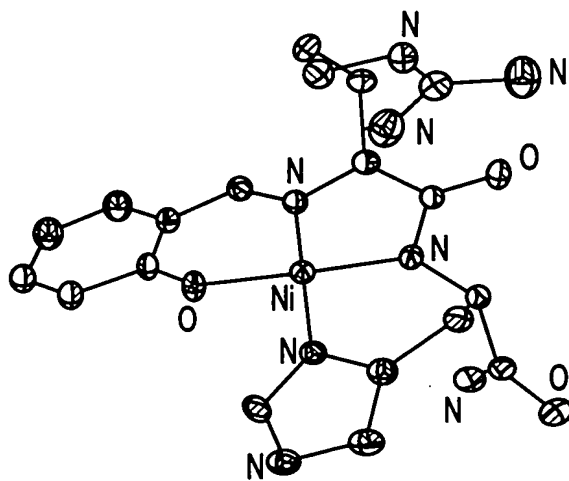


FIG. 5

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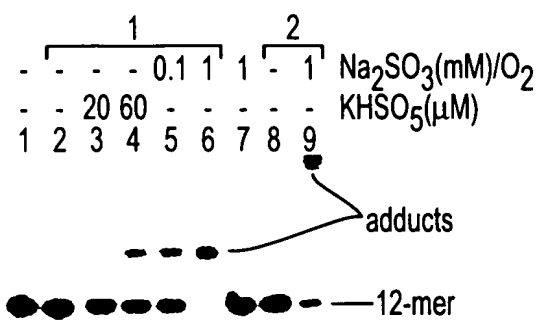


FIG. 6

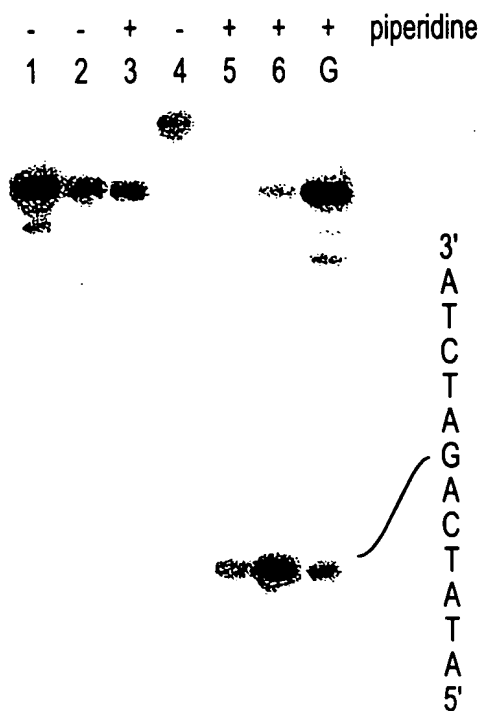


FIG. 7

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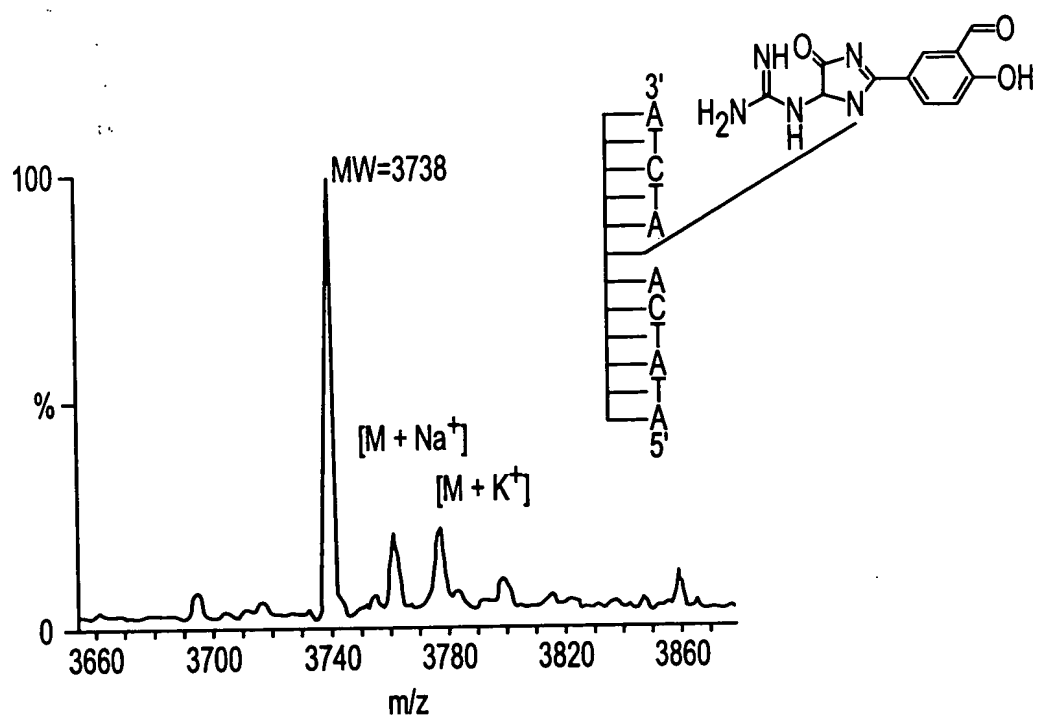


FIG. 8

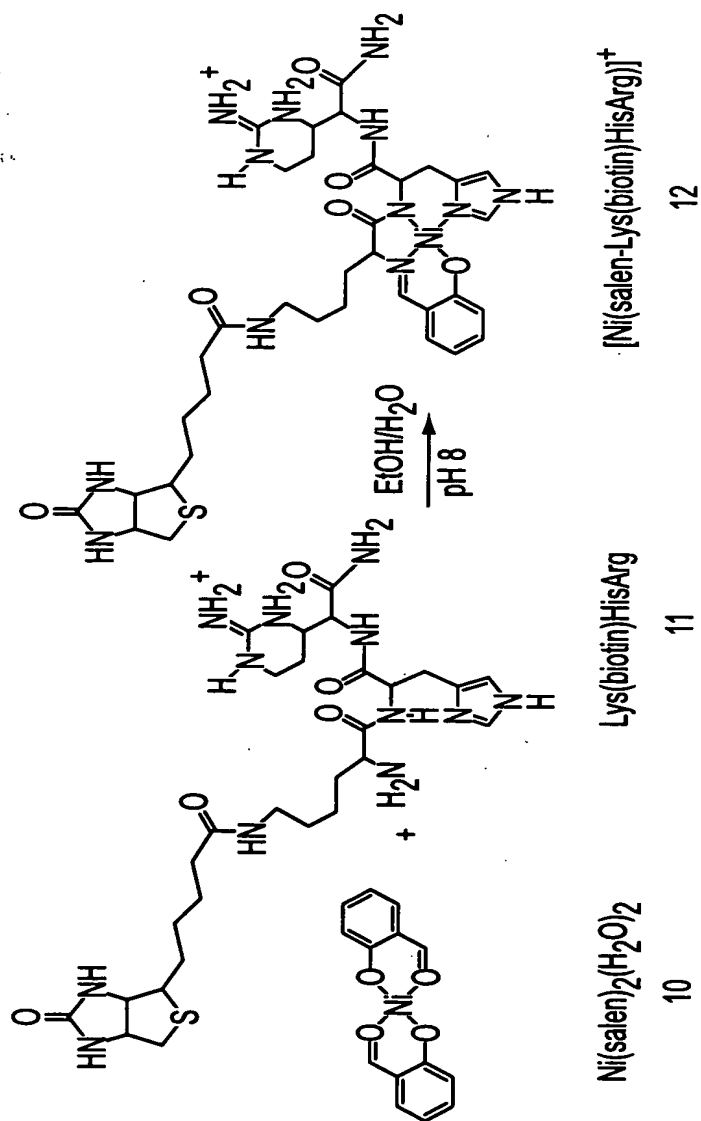


FIG. 9

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